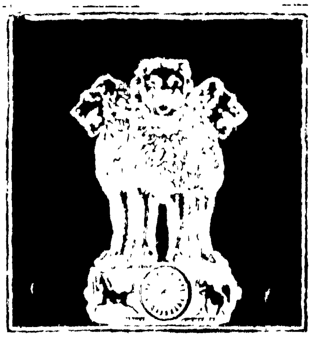

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CONTENTS

SERIES B VOL CXXIII

No. B 830—15 June 1937

PAGE

The Action of Radiations on Bacteria. III—γ-Rays on Growing and on Non-proliferating Bacteria. By D. E. Lea, R. B. Haines and C. A. Coulson. Communicated by Sir Frederick Hopkins, O.M., F.R.S. (Plate 1) 1

Pituitary Control of Alimentary Blood Flow and Secretion. Changes in the Stomach Produced by the Administration of Posterior Pituitary Extract. By E. C. Dodds, R. L. Noble, R. W. Scarff and P. C. Williams. Communicated by Sir Henry Dale, F.R.S. (Plates 2, 3) 22

Pituitary Control of Alimentary Blood Flow and Secretion. The Effect of Posterior Pituitary Extract on the Alimentary Secretions of Intact Animals. By W. C. Cutting, E. C. Dodds, R. L. Noble and P. C. Williams. Communicated by Sir Henry Dale, F.R.S. 27

Pituitary Control of Alimentary Blood Flow and Secretion. The Effect of Alterations in Blood Flow on Gastric Secretion. By W. C. Cutting, E. C. Dodds, R. L. Noble and P. C. Williams. Communicated by Sir Henry Dale, F.R.S. 39

Pituitary Control of Alimentary Blood Flow and Secretion. Gastric Secretion and Blood Flow in Hypophysectomized Animals. By W. C. Cutting, E. C. Dodds, R. L. Noble and P. C. Williams. Communicated by Sir Henry Dale, F.R.S. 49

A Study of the Medulla Oblongata of Cyprinodont Fishes with Special Reference to their Feeding Habits. By B. S. Bhimachar. Communicated by C. Tate Regan, F.R.S. 59

The Change of Visual Sensitivity with Time. By B. H. Crawford. Communicated by Sir John Parsons, F.R.S. 69

The Luminous Efficiency of Monochromatic Rays Entering the Eye Pupil at Different Points and a New Colour Effect. By W. S. Stiles. Communicated by Sir John Parsons, F.R.S. 90

No. B 831—1 July 1937

PAGE

The Linkage between the Genes for Colour-blindness and Haemophilia in Man. By Julia Bell and J. B. S. Haldane, F.R.S. 119

The Penetration into the Sea of Light of various Wave-lengths as Measured by Emission or Rectifier Photo-electric Cells. By H. H. Poole and W. R. G. Atkins, F.R.S. 151

The Connexions of the Arcuate Nucleus of the Thalamus. By W. E. Le Gros Clark, F.R.S. (Plates 4, 5) 166

On the Response of the Human Ear to Audio-frequency Electrical Stimulation. By C. S. Hallpike and H. Hartridge, F.R.S. (Plate 6) 177

Enzymes of some Wood-rotting *Polypores*. By S. R. Bose and S. N. Sarkar. Communicated by E. J. Butler, F.R.S. 193

Neoplasia following the Application of X-Rays to Inflammatory Lesions. By H. Burrows, W. V. Mayneord and J. E. Roberts. Communicated by E. L. Kennaway, F.R.S. (Plates 7, 8) 213

Experiments on Muscle Haemoglobin *in vivo*; the Instantaneous Measurement of Muscle Metabolism. By G. A. Millikan. Communicated by Sir Joseph Barcroft, F.R.S. (Plates 9–11) 218

No. B 832—3 August 1937

Respiration of Barley Plants. III—Protein Catabolism in Starving Leaves. By Edmund W. Yemm. Communicated by A. G. Tansley, F.R.S. 243

The Isolation and some Properties of Liquid Crystalline Substances from Solanaceous Plants infected with Three Strains of Tobacco Mosaic Virus. By F. C. Bawden and N. W. Pirie. Communicated by Sir Frederick Gowland Hopkins, O.M., F.R.S. (Plates 12, 13) . . 274

Analytic Studies in Plant Respiration. VII—Aerobic Respiration in Barley Seedlings and its Relation to Growth and Carbohydrate Supply. By H. R. Barnell. Communicated by F. F. Blackman, F.R.S. 321

PAGE

The Production of Cancer by Pure Hydrocarbons—IV. By W. E. Bachmann, J. W. Cook, A. Dansi, C. G. M. de Worms, G. A. D. Haslewood, C. L. Hewett and (Mrs.) A. M. Robinson. Communicated by E. L. Kennaway, F.R.S. (Plates 14, 15) 343

The Action of Nitrite on Haemoglobin in the Absence of Oxygen. By J. Brooks. Communicated by D. Keilin, F.R.S. 368

A Graphical Solution of a Differential Equation with application to Hill's Treatment of Nerve Excitation. By W. A. H. Rushton. Communicated by A. V. Hill, Sec. R.S. 382

No. B 833—17 August 1937

Discussion Meeting on The Transmission of Excitation in Living Material. By C. F. A. Pantin, F.R.S. 397

Quantitative Spectrographic Analysis of Biological Material—II. By J. S. Foster, F.R.S. and C. A. Horton. (Plate 16) 422

Heterosis and the Inheritance of Quantitative Characters. By Eric Ashby. Communicated by V. H. Blackman, F.R.S. 431

The Menstrual Cycle of the Primates. X—The Oestrone Threshold of the Uterus of the Rhesus Monkey. By S. Zuckerman. Communicated by W. E. Le Gros Clark, F.R.S. 441

The Menstrual Cycle of the Primates. XI—The part played by Oestrogenic Hormone in the Menstrual Cycle. By S. Zuckerman. Communicated by W. E. Le Gros Clark, F.R.S. (Plates 17, 18) . . . 457

The Mechanism of the Lateral Sense Organs of Fishes. By A. Sand. Communicated by J. Gray, F.R.S. (Plates 19–22) 472

The Sheath Components of the Giant Nerve Fibres of the Squid. By Richard S. Bear, Francis O. Schmitt and John Z. Young. Communicated by E. S. Goodrich, F.R.S. (Plate 23) 496

PAGE

The Ultrastructure of Nerve Axoplasm. By Richard S. Bear, Francis O. Schmitt and John Z. Young. Communicated by E. S. Goodrich, F.R.S. (Plate 24) 505

Investigations on the Protein Constituents of Nerve Axoplasm. By Richard S. Bear, Francis O. Schmitt and John Z. Young. Communicated by E. S. Goodrich, F.R.S. 520

Index 531

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The Action of Radiations on Bacteria

III—γ-Rays on Growing and on Non-proliferating Bacteria

By D. E. Lea, R. B. Haines and C. A. Coulson

Strangeways Research Laboratory and Low Temperature Research Station, Cambridge

(*Communicated by Sir Frederick Hopkins, O.M., F.R.S. Received* 8 *January* 1937)

[Plate 1]

1—Preamble

The authors' work (Lea, Haines and Coulson 1936)* on the action of α-rays and β-rays on bacteria was mainly concerned with tests of the validity of the target hypothesis. Experiments using γ-rays were commenced with the object of obtaining still further evidence on this question. While no essential difference is to be anticipated in the biological action of γ-rays and β-rays, the greater penetrating power of the former makes certain experiments possible which could not be carried out with β-rays. Thus, in a disinfection experiment lasting several hours, it is convenient to be able to expose a relatively large quantity of liquid and to follow the course of disinfection by taking samples at intervals. Further, it is possible by the same method to observe the influence of radiation upon the growth of bacteria in a liquid nutrient medium. This last type of experiment seemed particularly suitable as a test of the target theory, since on that theory one would expect growth in the medium, and death produced by the radiation, to take place independently and at their normal rates, a result not to be anticipated on other theories. Actually, however, the issue was found to be complicated by the fact that the radiation, in addition to its lethal action, had the effect of causing the production of organisms many times longer than the normal. The present paper, therefore, is concerned with an investigation of these long forms.

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* Subsequently referred to as Part I and Part II.

2—THE γ-RAY SOURCE

Approximately 920 mg. of radium were available in various containers; the filtration used was sufficient to ensure that only the γ-rays reached the biological material. Owing to the necessity of using the radium in containers designed for other experiments, and of working at short distances in order to obtain a sufficiently high intensity, the radiation was by no means of uniform intensity throughout the tube of liquid exposed. Mixing by convection currents, and during the process of sampling, was probably sufficient to ensure that over an extended time each organism received a dose of radiation corresponding to the average intensity over the whole volume of liquid. This average intensity was measured with the aid of a cylindrical ionization chamber of graphite, having a size approximating to that of the exposed tube. The ionization current was measured with an instrument recently described (Lea 1937).

3—THE IRRADIATION OF NON-PROLIFERATING BACTERIA

Experiments were made with *B. coli communis* Escherich and with spores of *B. mesentericus* and *B. megatherium*. The organisms were prepared in suspension in glass-distilled, sterile water as described in Part II, p. 65. Tubes containing 3 c.c. of the liquid were irradiated and samples taken at intervals by means of calibrated dropping pipettes. The samples were suitably diluted and fractions plated in the customary manner. In figs. 1 and 2 are plotted the logarithms of the surviving fractions as a function of the time of irradiation. The survival curves are exponential. It is difficult to obtain accurate results for the spores on account of the very slow rate of death. In calculating the rate of death due to the radiation, correction is made where necessary for the (natural) death shown by the controls.

In Table I are given the half-value times, i.e. the times required to kill half the number of organisms. The mean lethal doses (m.l.d.) also listed are obtained by calculating the doses administered in a time $T/\log_e 2$, where T is the half-value time. The intensity of radiation in these experiments was $1500r$ units per hour. For comparison, Table I reproduces also the data for β-rays given in Part II. In view of the indirectness of the method by which the β-ray half-value times were converted to ionization doses (Part II, p. 71), the difference between the mean lethal doses for β-rays and γ-rays is not considered significant. It thus appears that the mean lethal dose is sensibly independent of intensity over a range of more than 100 : 1, and independent of temperature between 0 and 37° C.

The concentration of organisms in the suspensions used in these experiments was of the order of 10^6/c.c. Experiments have been performed with *B. coli* in which the concentration has been varied between 10^4 and 10^9/c.c. without obtaining any indication of a dependence of rate of death upon concentration.

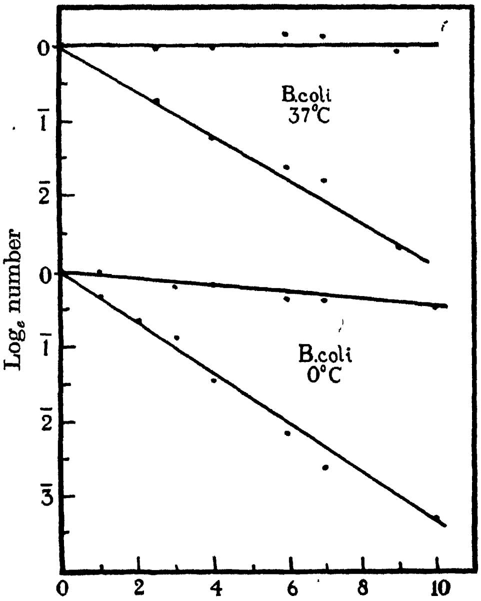


FIG. 1

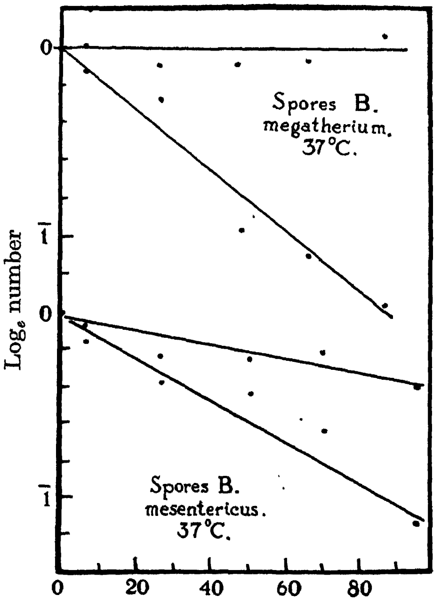


FIG. 2

Irradiation of bacteria in aqueous suspension with γ-rays.

TABLE I—IRRADIATION OF RESTING BACTERIA

	γ-rays			β-rays		
Organism	Temp. ° C.	Half-value time hr.	m.l.d. (r units)	Temp. ° C.	Half-value time min.	m.l.d. (r units)
B. coli	37	2·4	$5{\cdot}1 \times 10^3$	20	3	4×10^3
B. coli	0	2·4	$5{\cdot}1 \times 10^3$	—	—	—
B. mesentericus spores	37	100	2×10^5	20	22	$1{\cdot}1 \times 10^5$
B. megatherium spores	37	40	9×10^4	—	—	—

A test has been made to decide whether the lethal action of the radiation is a direct action upon the organisms or is a secondary action due to some chemical change in the water produced by the radiation. Sterile water was irradiated for a period of several hours, and then removed from the radium and inoculated with *B. coli*. Samples were taken at intervals and plated. No death was detectable during the first 10 hr. following inoculation, but in some of the experiments death began to occur after about 10 or 15 hr. had elapsed. Over these long periods, however, death occurred in the controls also, and it was not established with any certainty that there was an effect to be attributed to the action of the radiation upon the water. If such effect existed, it was negligible for experiments lasting for less than 10 hr.

4—THE IRRADIATION OF GROWING BACTERIA

In these experiments a tube of a liquid nutrient medium was inoculated and exposed to the radiation. In some instances the irradiation was commenced during the lag phase, before division was in progress, in others it was commenced during the phase of logarithmic growth. Samples were taken at intervals from the irradiated tube and also from the control tube, and plated. The general character of the results may be seen from fig. 3, which is a composite curve embodying the results of two experiments, in one of which irradiation was commenced in the lag phase, and in the other in the logarithmic phase. It is seen that the number of viable organisms in the exposed tube diminishes roughly exponentially at a rate which is smaller the greater the rate of growth of the controls. In experiments in which the controls are multiplying sufficiently rapidly, the viable population of the exposed tube increases, though at a slower rate.

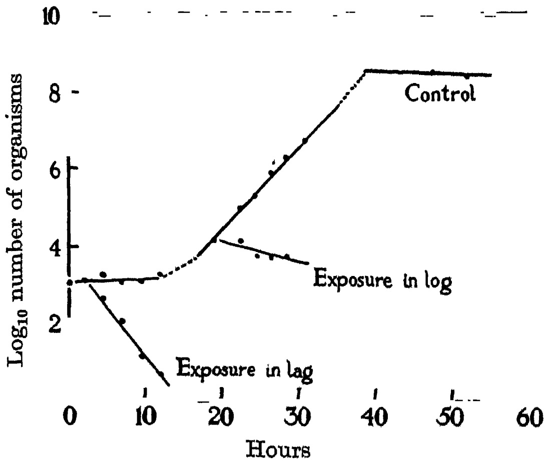


FIG. 3—*B. coli* in Nelson's medium.

The change of population in the control tube may be represented by the formula $n = n_0 e^{\alpha t}$, where n is the population per c.c. at time t and n_0 its value at time $t = 0$. α is a constant in the logarithmic phase, and takes the value 0 in the lag phase. The change of viable population in the exposed tube may similarly be represented by $n = n_0 e^{\epsilon t}$, ϵ being negative when the viable population is decreasing. In Table II, data are given relating to a number of experiments in which a variety of temperatures and media were used and several different values of α consequently obtained.

TABLE II—IRRADIATION IN NUTRIENT MEDIA

Exp.	Medium	Temp. ° C.	Phase	Growth rate: Control (α)	Growth rate: Exposure (ϵ)	($\alpha-\epsilon$)
71	Nelson*	37	Log	+0·48	+0·02	0·46
75	,,	37	,,	+0·48	−0·14	0·62
73	,,	37	Lag	+0·02	−0·57	0·59
74	,,	37	,,	−0·02	−0·46	0·44
72	Broth	37	Log	+1·47	+0·97	0·50
77	,,	37	,,	+1·47	+0·99	0·48
77	,,	37	Lag	−0·69	−1·15	0·46
95	,,	23	Log	+0·44	0·0	0·44
102	,,	13	,,	+0·05	−0·34	0·39
					Mean value	0·49

* (Nelson 1926.)

There appears to be no systematic variation of $\alpha - \epsilon$, while α varies between −0·69 and +1·47.

The constancy of $\alpha - \epsilon$ may be explained on the assumption that cell division and death are independent, division proceeding at the same rate in the exposed medium as in the controls, and death proceeding at the same rate in the presence of nutriment as in its absence. When bacteria are multiplying in the absence of radiation, their number may be represented by the formula $n = n_0 e^{\alpha t}$. It follows that the increase of the number in a short interval of time is given by the formula $dn = n\alpha dt$. When bacteria are being killed by radiation in the absence of nutriment the number at any time is represented by the formula $n = n_0 e^{-\gamma t}$, whence it follows that the decrease of the number of viable organisms in a short interval of time is $-dn = n\gamma dt$. Supposing multiplication and death to be occurring simultaneously, the increase in the number of viable organisms in time dt is $dn = n(\alpha - \gamma)\, dt$, whence one deduces that the number at any time is given by the formula $n = n_0 e^{(\alpha-\gamma)t}$. Since this same number was represented above by $n = n_0 e^{\epsilon t}$, one has $\alpha - \epsilon = \gamma$. Thus $\alpha - \epsilon$ should be constant.

The mean value of $\alpha - \epsilon$ in Table II (0·49) does not agree with the value of $\gamma = 0{\cdot}29$ deduced from the half-value period of 2·4 hr. given for *B. coli* irradiation in aqueous suspension in Table I.

The authors found it difficult to obtain constantly reproducible results in this type of experiment, the behaviour of the bacteria being very sensitive to environmental and other factors difficult to control. In particular it seems doubtful whether this strain of *B. coli* growing in Nelson's medium can be regarded as physiologically "normal", since if repeatedly subcultured in this medium it tended to lose its motility and to grow less well. The results collected in Table II, however, are believed to be mutually consistent, since they were obtained in a series of experiments performed within a fairly short space of time and with the same batch of culture media.

5—THE PRODUCTION OF LONG FORMS

Microscopic examination in connexion with the experiments listed in Table II showed that in certain instances abnormally large organisms appeared in the exposed tubes. The abnormal forms were not produced to any great extent in Nelson's medium, nor much in broth at temperatures such that the rate of growth of the controls was either very rapid or very slow. At intermediate temperatures, e.g. 23° C., however, long forms appeared freely in broth after a few hours' irradiation. Wilson (1906) has shown that long, filamentous forms of *B. coli* are produced by certain chemicals in the culture medium. Owing to the known variability of bacterial morphology, precautions have been taken to make the present experiments as critical as possible. We first observed the phenomenon with a strain of *B. coli* Escherich, Lister No. 86. In any stock subculture of this organism a few organisms could always be found two or three times the normal length. To eliminate the possibility of contaminants, or selection of an already existing abnormality, a single-cell subculture, taking an organism of normal length, was made with the aid of the Chambers micromanipulator. This subculture had the same biochemical properties as the original, and exhibited the same phenomenon on irradiation. Having established the fact that abnormal forms of *B. coli* are produced from a single-cell culture of undoubted purity, it was shown that the effect is probably a general one by irradiation of a number of other organisms, namely *B. coli* Houston, Lister No. 691, *B. pyocyaneus* Lister No. 2005, *B. fluorescens liquefaciens* Lister No. 964, and a strain of *Achromobacter* previously isolated and described by one of us (Haines 1933). The detailed experiments have been performed throughout using *B. coli* Houston with 12–24 hr. subcultures, and the same batch of nutrient broth.

Photographs of some of the abnormally long forms obtained are shown in Plate 1. It is interesting to note that the growth on agar, after long periods of irradiation leading to the production of very long forms, has changed macroscopically. In place of the normal smooth film, the growth has a rhizoid appearance (Plate 1).

Measurements have been made of the lengths of some thousands of these long forms, with the aid of a Ramsden micrometer eyepiece used in conjunction with a $\frac{1}{12}$ in. oil-immersion objective and a darkground condenser. Fig. 4 is an example of the type of result obtained. A tube of broth was inoculated with *B. coli* and incubated for 10·5 hr. at 22° C., by which time the middle of the logarithmic phase had been reached, the concentration of organisms being about 3×10^6/c.c. Two portions of the broth were now taken, one being exposed to γ-radiation and the other acting as a control, the temperature remaining at 22° C. The γ-ray intensity was $2100r$ units/hr. Samples were taken at intervals from the two tubes and measurements were made of the lengths of fifty organisms. The mean lengths are plotted in fig. 4 as a function of the time of irradiation. Fig. 5 shows the frequency of occurrence of different lengths after 4·2 hr. irradiation. The lengths are plotted as abscissae, the unit of length being the mean length of the unirradiated organisms. The ordinate corresponding to an abscissa l is the

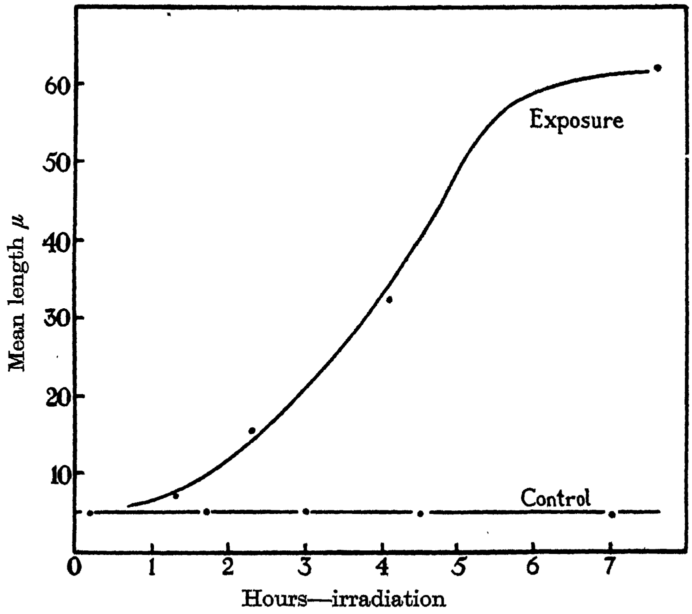


Fig. 4—Production of long forms of *B. coli*.

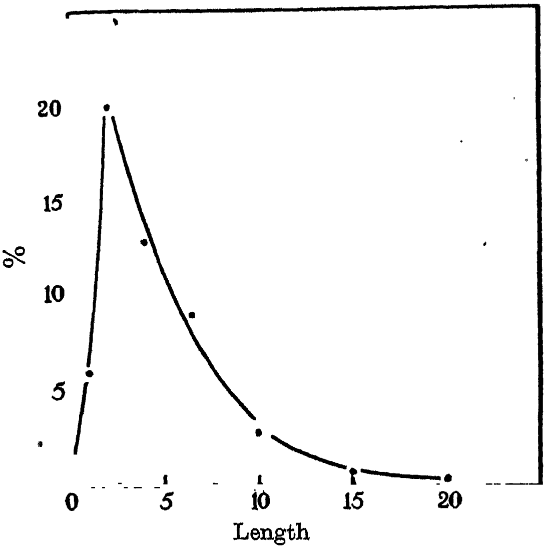


FIG. 5—Length distribution of long forms.

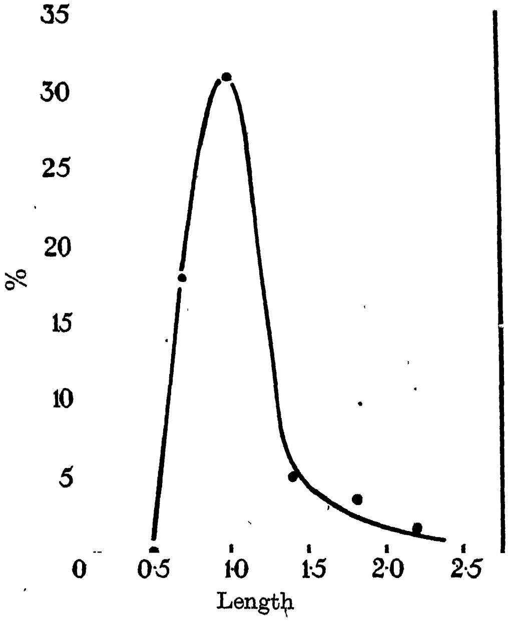


FIG. 6—Length distribution of normal forms.

percentage of organisms having a length between $l-0{\cdot}5$ and $l+0{\cdot}5$. Fig. 6 shows the frequency of occurrence of different lengths of organism in the unirradiated controls. The ordinate corresponding to an abscissa l is the percentage of forms having a length between $l-0{\cdot}1$ and $l+0{\cdot}1$. It is seen that only a very small portion of the normal forms have lengths greater than twice the mean length.

6—THE NATURE OF THE LONG FORMS

It appears *a priori* that a long form must be either

(*a*) a new organism or "mutation" produced by the action of radiation, or

(*b*) a chain of normal organisms, or

(*c*) a single long organism having the same radio-sensitivity as a normal form.

With other organisms mutations are known to occur under the action of radiation, and Spencer (1935), who has obtained long forms by irradiating the bacteria with β-rays and γ-rays, is of the opinion that mutations do occur when the organisms are exposed to radiation continuously through a number of subcultures. However, it has been established that the long forms produced in the present experiments are not mutations. When a long form is plated out on agar it produces a colony of individuals having the appearance, macroscopic and microscopic, and exhibiting the biochemical reactions characteristic of normal *B. coli.*

To decide between the alternatives (*b*) and (*c*) is more difficult. The long forms have been carefully examined under the microscope, both unstained using dark ground illumination, and stained by a variety of methods. Often an organism is clearly seen to consist of two or three segments. This is not unusual in broth, and is exhibited to about the same extent by the unirradiated *B. coli.* Inside the individual segments, however, which may be many times the length of a normal organism, no indication of a chain structure has been observed. Occasionally, particularly after standing for some hours after removal from the radiation, a long form has been observed which has thirty or forty segments and obviously is a chain of normal individuals. This suggests that possibly the long forms are capable of breaking up into chains of normal individuals. The ease with which the chain structure may be distinguished in these cases makes it unlikely that it exists in those organisms in which it has not actually been observed. Vigorous shaking has failed to break up the long forms.

There does, however, seem to be a difference in the motility of normal and abnormal forms. Whereas a chain of three individuals of normal *B. coli* shows the characteristic, erratic, "darting" motion due to flagellar propulsion, the long forms exhibit a curious, sinuous, undulating motion, similar to that shown by some strains of *B. proteus*. This occurs sometimes even when the long form is visibly segmented. Occasionally the segments could be seen breaking off the longer cell. Segments of almost any length were observed in the irradiated cultures, while in the chains of two or three cells sometimes obtained in normal cultures, the segments were of equal length and generally slightly shorter than the free-swimming individual. Long forms up to about one hundred times the normal length have been obtained in broth, and very much longer forms still on agar. The latter are not motile when suspended in water. No appreciable change in the diameter of the cell seems to take place on irradiation. Certain long forms after prolonged periods of irradiation lose their motility and become markedly granular, and in time can be seen disintegrating, the cell wall disappearing and leaving a mass of granules throughout the space occupied by the cellular protoplasm.

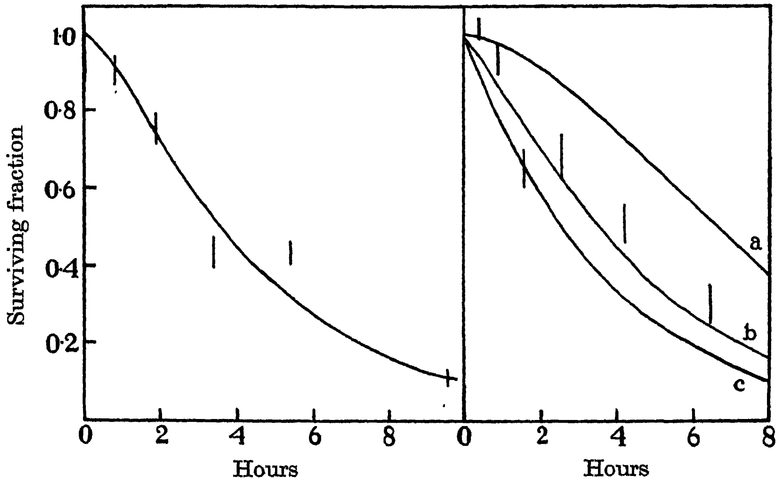
The most promising method of deciding whether the long forms are chains of individuals or single large organisms is to compare the survival curves obtained by exposing them to radiation with the survival curve for normal individuals. If a long form is a chain of individuals it will give rise to a colony when plated, even if all the individuals of the chain except one have been killed. The time required to kill all the individuals of the chain when exposed to radiation considerably exceeds the time to kill a single organism, and thus the survival curve for a long chain will be much less steep than for a normal individual. If the survival curve of a normal individual is represented as a function of the time by the formula $n = n_0 e^{-\gamma t}$, it is easy to show (Glocker 1934) that the survival curve of a chain of m individuals will be:

$$n = n_0\{1-(1-e^{-\gamma t})^m\}. \tag{A}$$

When testing the theory that the long forms are chains of normal individuals, the value of m is taken to be the ratio of the length of a long form to the average length of a normal form. Since the long forms exhibit a wide variation of length, it is necessary to determine a distribution curve such as fig. 5, and to calculate the survival curve by summing in appropriate proportions a series of expressions such as equation (A) with different values of m.

To determine the coefficient γ, experiments are made with normal forms grown under conditions similar to the long forms except for the absence of radiation. Grown in this way, some of the normal forms can be seen under the microscope to have two or more segments, and it is necessary to take this factor into account in deducing γ from the experimental survival curves.

In the first series of experiments long forms were produced by inoculating a tube of broth, incubating at 23° C. for 8 hr., and then irradiating at an intensity of 1500*r* units/hr. for 4 hr. at the same temperature. After completion of the irradiation the broth was centrifuged and decanted, the organisms after washing being finally made up into a suspension in water, aerated and vigorously shaken to separate the clumps which had formed during centrifuging. The aqueous suspension was then irradiated at 23° C. at an intensity of 2100*r* units/hr. and the survival curve plotted by plating samples in the usual manner. Microscopic observations were also made on the aqueous suspension to obtain the distribution of length and the distribution of numbers of visible segments.



Curves theoretical, points experimental.

FIG. 7—Normal forms. FIG. 8—Long forms.

The control experiments with normal forms followed the same procedure, with the omission of the irradiation while growing. The distribution of segments among the controls was

Segments	1	2	3	4
% of forms	46	50	3	1

The experimental points on the survival curve of the normal forms are shown in fig. 7. The theoretical curve is calculated as described, taking the above segment distribution into account and using the value $\gamma = 0{\cdot}28$ which by trial is found to give the best fit of the experimental points. This value of γ is now used to calculate the theoretical curves of fig. 8 which refer to the long forms. The three curves assume respectively:

(*a*) each long form consists of a chain of normal ones, the number of individuals in the chain being equal to the ratio of the length of a long form to the mean length of a single-segment normal form,

(*b*) each long form consists of as many individuals as it has visible segments,

(*c*) the long forms behave as single individuals having the same rate of death as single-segment normal forms.

The segment distribution needed to calculate (*b*) was determined by microscopic observation to be

Segments	1	2	3	4
% of forms	55	35	7	3

while the length distribution needed to calculate (*a*) was similarly determined as

Length l*	1	2	4	8	16	32
% of forms	8	25	40	20	4	3

It is evident that the curve (*b*) best fits the experimental points, and that the long forms therefore consist only of as many individuals as there are visible segments. Five separate experiments using the long forms and three using the normal forms were performed, and the experimental points in figs. 7 and 8 are mean values, the errors indicated being standard deviations deduced from the mutual consistency of the observations.

In addition an experiment was performed in which the organisms grown in broth were not centrifuged at the completion of the growth period but were simply cooled to 0° C. to prevent further division, and the survival curve obtained by irradiation at that temperature. The survival curve of the normal forms was found to be fitted by the same value $\gamma = 0{\cdot}28$ as in the previous experiment. (It has already been shown that the process of disinfection with γ-rays has no temperature coefficient.) Figs. 9 and 10 for this experiment correspond to figs. 7 and 8 for the former experiment, and the conclusion is seen to be the same. The distributions of lengths and of segments were rather similar to those quoted above.

A third variation of this experiment was performed, in which the long forms were produced by irradiation during growth upon the surface of nutrient agar. The agar was contained in a Petri dish and was inoculated by flooding the surface with an aqueous suspension of *B. coli*. After incubation for 4 hr. at 23° C. the Petri dish was placed over a source of

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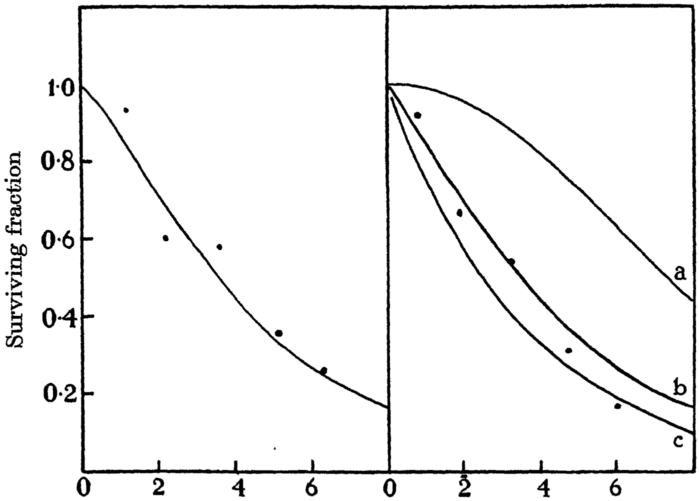
* The length listed as l includes all forms in the range $l\left(1-\frac{1}{\sqrt{2}}\right)$ to $l\left(1+\frac{1}{\sqrt{2}}\right)$, the unit of length being the mean length of a single segment of a normal form.

radium (760 mg.), the intensity at the surface of the agar in the centre of the dish being 2000r units/hr., and left for a further 8·7 hr. at the same temperature. The growth was then scraped off the agar in the centre of the dish, emulsified in water and shaken vigorously. A microscopic examination was made to determine the distribution of total length and of number of segments in the long forms with the results:



Curves theoretical, points experimental

FIG. 9—Normal forms. FIG. 10—Long forms.

Segments	1	2	3	4	5	6	9	30
% of forms	83	7	5	1	1	1	1	1

Length l*	1	2	4	8	16	32	64
% of forms	0	6	10	16	20	45	3

The unit of length is 4·2μ, the average length of a single segment normal form. The normal forms grown on an agar slant showed little segmentation:

Segments	1	2
% of forms	92	8

The value of γ required to fit the experimental survival curve (fig. 11) was 0·67. Using this value of γ, the three theoretical curves (*a*), (*b*), (*c*) of fig. 12 were calculated, and once again it is seen that the experimental points for long forms lie nearest to the curve (*b*). It is remarkable that the rates of disinfection of normal forms exposed to the same intensity of γ-radiation are different for *B. coli* grown on agar ($\gamma = 0{\cdot}67$) and *B. coli* grown in broth ($\gamma=0{\cdot}28$).

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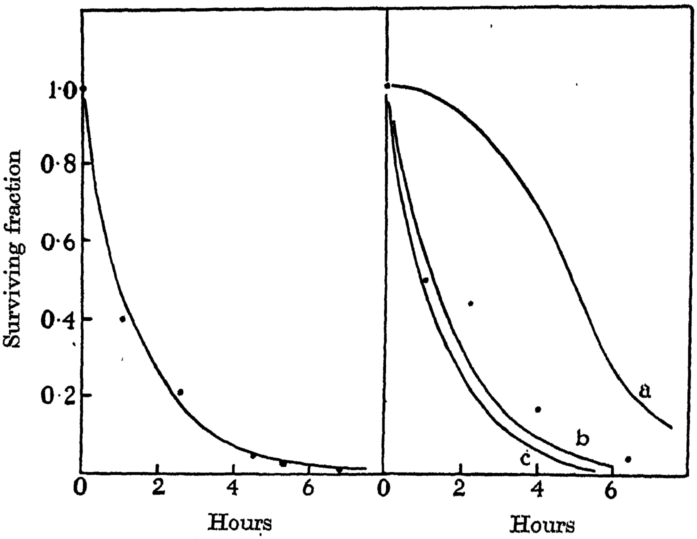
* The length listed as l includes all forms in the range $l\left(1-\frac{1}{\sqrt{2}}\right)$ to $l\left(1+\frac{1}{\sqrt{2}}\right)$.



Curves theoretical, points experimental

FIG. 11—Normal forms. FIG. 12—Long forms.

7—THE THEORY OF THE PRODUCTION OF LONG FORMS

It has been seen in the last paragraph that the radiation method of deciding whether the long forms are chains of individuals or single organisms suggests the latter alternative. It must be emphasized, however, that the theoretical curves (*c*) in figs. 8, 10 and 12 have been calculated on the assumption that the individuals composing a chain would separately have the same sensitivity to radiation as the free normal forms. If, on the contrary, the mean lethal dose for individuals of a chain were only half as great as for free normal organisms, the curve (*a*) would not be sufficiently distinguishable from the curves (*c*) and (*b*) for the test to be applied. The possibility of such a coincidence nullifying the conclusions of the last paragraph should not be overlooked, but for the remainder of this paper those conclusions will be accepted.

It appears then that under suitable conditions a culture of *B. coli* growing on agar or in broth in the presence of radiation is capable of producing long forms, and that these long forms, or rather the visibly separate segments, are single giant individuals having the same rate of death under irradiation as the normal forms. This production of long forms must come about through a disturbance of the balance between increase of cell volume and division which normally exists in active cultures. Experiments have been made at 23° C. in broth cultures exposed to an intensity of radiation of 2100*r* units/hr. which show that division under these conditions is almost completely inhibited. The method was to make direct microscopic ("total") counts of the number of organisms per c.c. with the aid of a Thoma-Hawksley counting chamber (Wilson 1922) 0·02 mm. deep and divided into squares of side 0·05 mm. A $\frac{1}{7}$ in. oil-immersion objective was used in conjunction with a dark ground condenser. In each experiment a tube of broth was inoculated with *B. coli* and incubated for from 9 to 10·5 hr. at 23° C., by which time the count was between 10^6 and 10^7/c.c. Irradiation was then commenced and samples taken at intervals and counted. The results given are the means of three separate experiments. In fig. 13, the logarithms of the ratios of the total counts to the initial total counts are plotted as a function of the duration of the irradiation. The rate of increase of total count is negligible by comparison with the corresponding controls, which double approximately every 1·4 hr.

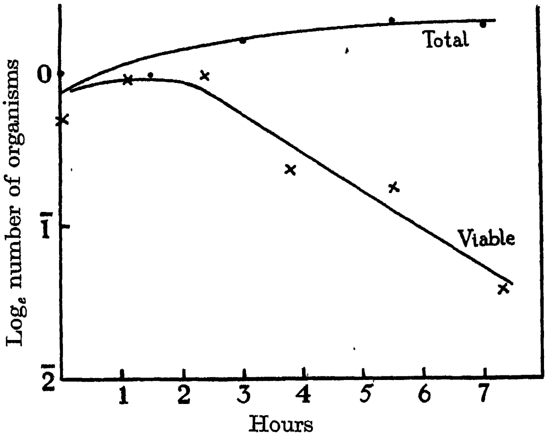


FIG. 13—Total and viable counts, *B. coli* in broth irradiated with γ-rays.

In the same experiments the viable counts were also plotted by sampling and plating in the usual way. Fig. 13 also shows the logarithm of the ratio of the viable count to the initial total count as a function of the duration of the irradiation. The gradient of the straight portion of the viable curve in fig. 13 is 0·25, indicating that the viable count is represented by the formula $e^{-\gamma t}$ where γ has the value 0·25. This is in fair agreement with the experimental points of figs. 7–10, which may be represented approximately by $e^{-\gamma t}$ with $\gamma = 0{\cdot}20$. Thus the rate of diminution of the viable count is the same when growth, in the sense of increase of cell volume, is occurring as it is in its absence, showing that the lethal action of the radiation is independent of the growth rate.

It appears from fig. 13 that during the early stages the viable count does not diminish according to the formula $e^{-\gamma t}$ adhered to later in the course of the irradiation, but remains approximately constant, or perhaps even increases. This may be experimental error; on the other hand, it is quite possible that the inhibition of division does not follow instantaneously upon the commencement of the irradiation, but may require some time to take effect.

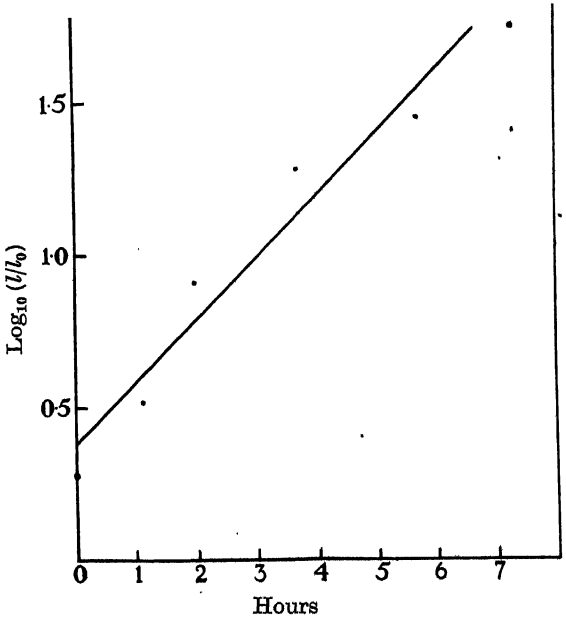


FIG. 14—Maximum size of long forms.

It appears then from a study of the viable and total counts in the exposure that the radiation under the conditions of these experiments is able to inhibit division almost completely. It remains to be discussed whether it has any effect upon the rate of increase of volume of the viable organisms present in the irradiated broth at various intervals. The longest organisms present at any period are presumably those which have not yet been killed by the radiation. Fig. 14 shows the result of this experiment. The quantity plotted as ordinate is the logarithm of the ratio of the length of the longest

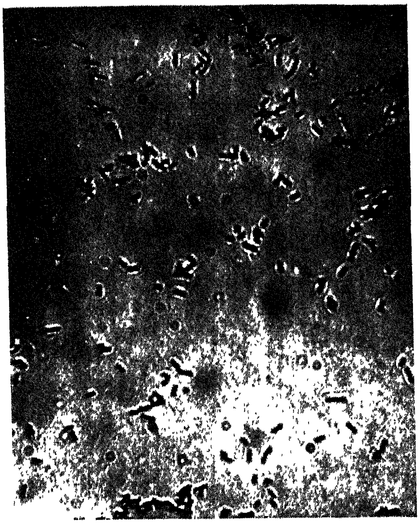


FIG. 18—*B. coli* Houston. Normal cells 24 hrs. on agar at 23° C. Carbol fuchsin. × 1200.

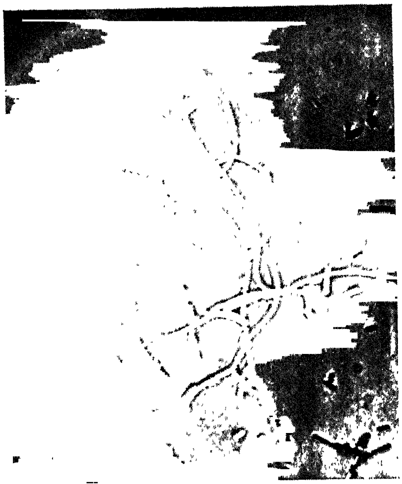


FIG. 19—*B. coli* Houston. 12 hrs. agar 23° C., irradiated 24 hrs.

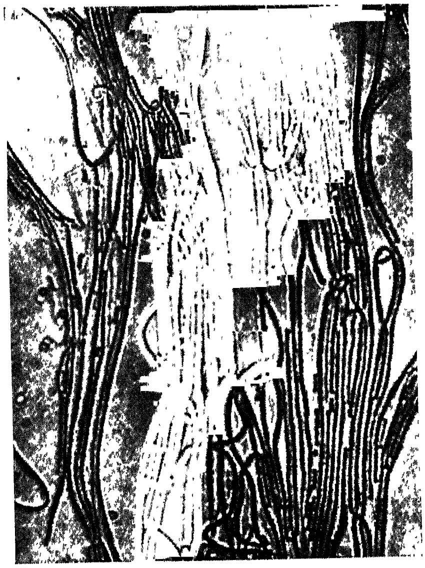


FIG. 20—*B. coli* Houston. Grown agar 2½ hrs. 23° C. irradiated 21 hrs. Methylene blue. × 1200.

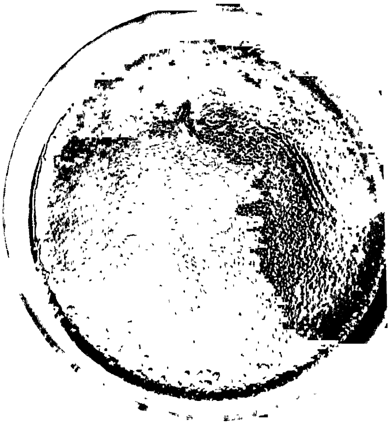


FIG. 21—*B. coli* Houston. Rhizoid growth on agar after 21 hrs. irradiation at 23° C. Plate from which organisms in fig. 20 were taken. Approximately actual size.

organism to the mean length of the organism before irradiation. The longest organism for a particular exposure time was selected by measuring about 150 organisms chosen at random. The length of the longest organism is seen to be an exponentially increasing function of the time, being represented by the formula $e^{\alpha t}$, where $\alpha = 0{\cdot}47$.

This figure is to be compared with the corresponding rate of volume increase for the unirradiated controls. The mean length of the control organisms was measured at intervals. Little change was found, there being a barely perceptible tendency for the mean length to diminish. The total number of organisms per c.c. in the control tubes was also determined at intervals. The product of mean length and total count gave the value of the total length of all the organisms per c.c. in the unirradiated broth, and this is plotted as a function of the time in fig. 15. The total length is seen to be an exponentially increasing function of the time, following the formula $e^{\alpha t}$ where $\alpha = 0{\cdot}51$.

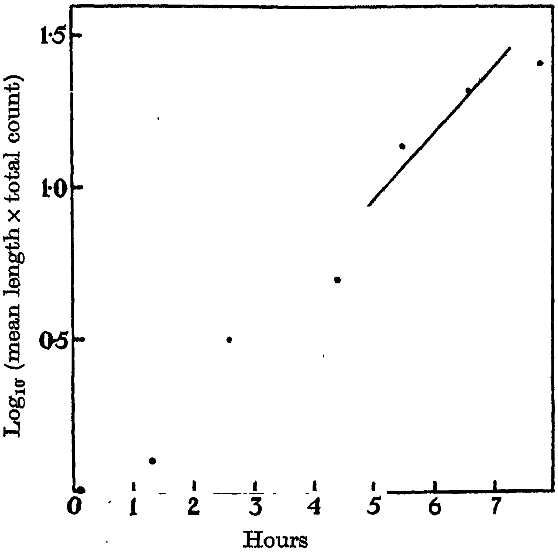


FIG. 15—Total length of controls..

This value agrees with the coefficient α for the long forms within the accuracy of the experiments.

The following picture of the process of production of long forms is thus suggested. The organisms in the irradiated broth absorb nutriment and increase in volume at a rate unaffected by the radiation, without however dividing, so that the lengths of the organisms rapidly increase. From time to time an organism is killed outright by the radiation, this lethal action taking place at random with equal probability for a long or for a short form. After a given time therefore a variety of lengths are present, ranging from the initial length of the organism up to a maximum. The shortest forms are those which have been killed early, the longest are those which have survived for the greatest time. It has been seen that this picture of the process is in agreement with measurements made of the variation with time of the total count, of the viable count, and of the maximum length of the organisms. It is of interest to see also whether it is able to predict accurately the frequency distribution of the lengths of the organisms after various periods of irradiation. The calculation is made on the basis of the following assumptions:

(i) Increase of length, without division, takes place according to the formula $l = l_0 e^{\alpha t}$, where l_0 is the initial length and l the length after time t. α is deduced from fig. 15 to be 0·51.

(ii) All forms are equally sensitive to the radiation, the surviving fraction being given as a function of the time of irradiation by the formula $e^{-\gamma t}$. Actually, on account of the existence of a certain amount of segmentation the survival curve is theoretically not strictly exponential, but an exponential curve with $\gamma = 0{\cdot}20$ sufficiently nearly fits the experimental results shown in figs. 7–10.

Assuming that at the commencement of the irradiation the organisms are of a uniform length l_0, then at any subsequent time those having a length l are those which have survived a time T given by $l = l_0 e^{\alpha T}$. But the proportion of organisms surviving a time T is $e^{-\gamma T}$. Thus if, at a time t, P is the proportion of organisms having a length $\geqslant l$, then $P = \left(\frac{l}{l_0}\right)^{-\gamma/\alpha}$ with the further condition that $P = 0$ for $l > l_0 e^{\alpha t}$. Using the values $\alpha = 0{\cdot}51$, $\gamma = 0{\cdot}20$, one obtains:

$$\text{for} \qquad 1 \leqslant \frac{l}{l_0} \leqslant e^{0\cdot 51 t} \quad P = \left(\frac{l}{l_0}\right)^{-0\cdot 39}$$

$$\text{and for} \qquad \frac{l}{l_0} > e^{0\cdot 51 t} \quad P = 0.$$

In fig. 16, P is plotted as ordinate with $\log_e(l/l_0)$ as abscissa for irradiation times of 0–10 hr.

Before comparison is made between theory and experiment it is desirable to take into account the fact that initially the organisms are not all of equal length, but show a distribution about the mean of the type illustrated in fig. 6. The organisms used in the experiments on length distribution about to be described had initially a mean length of $l_1 = 5{\cdot}2\mu$. For the purposes of calculation the organisms were divided into six groups, the mean length of the organisms in each group and the proportion of the total number of organisms residing in each group being:

$\log_e(l/l_1)$*	$\bar{1}\cdot5$	$\bar{1}\cdot7$	$\bar{1}\cdot9$	0·1	0·3	0·5
% of forms	2	31	22	36	7	2

The curves of P as a function of l/l_1 were calculated for the six groups and summed in the proportions listed above. The calculation was effected for the times 0, 1·1, 2·0, 3·7, 5·7 and 7·3 hr. for which experimental data were available. The curves of fig. 17 show the results of these calculations. The ordinate of any point of a curve represents the proportion of organisms having lengths greater than (or equal to) l, the abscissa being the value of $\log_e(l/l_1)$, where l_1 is the mean initial length $5\cdot2\mu$. Also shown in fig. 17 are the experimentally determined length distributions. At each of the six times for which data are given, from 150 to 300 bacteria were measured. The agreement between the experimental points and the theoretical curves is considered satisfactory for work of this nature. Additional support is thus obtained for the mechanism of the production of long forms postulated in this section.

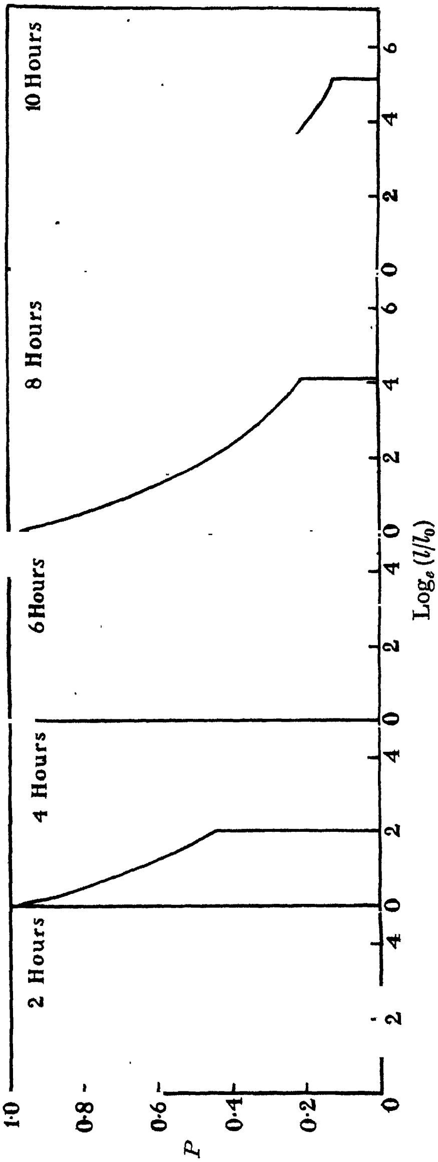


FIG. 16—Length distributions (theoretical).

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* Under $\log_e(l/l_1)$ are included all forms in the range ± 0·1 about this value.

8—The Inhibition of Division by Irradiation

It appears from the foregoing that in addition to and independently of its lethal action, radiation is able to exercise an inhibitory effect upon division. It is proposed to make the detailed investigation of this inhibitory action the subject of further work, and only an incomplete discussion can be attempted now. The inhibitory action is temporary, and the organisms which have been inhibited are able to divide and produce normal individuals when removed from the radiation and transplanted into fresh medium. Whether the action be direct, or indirect through some change in the nutrient medium, it appears that under the conditions of the experiments described, division is fairly completely inhibited by a dose of radiation only sufficient to produce death in a relatively small proportion of the organisms. The inhibition of division by a dose of radiation smaller than the lethal dose

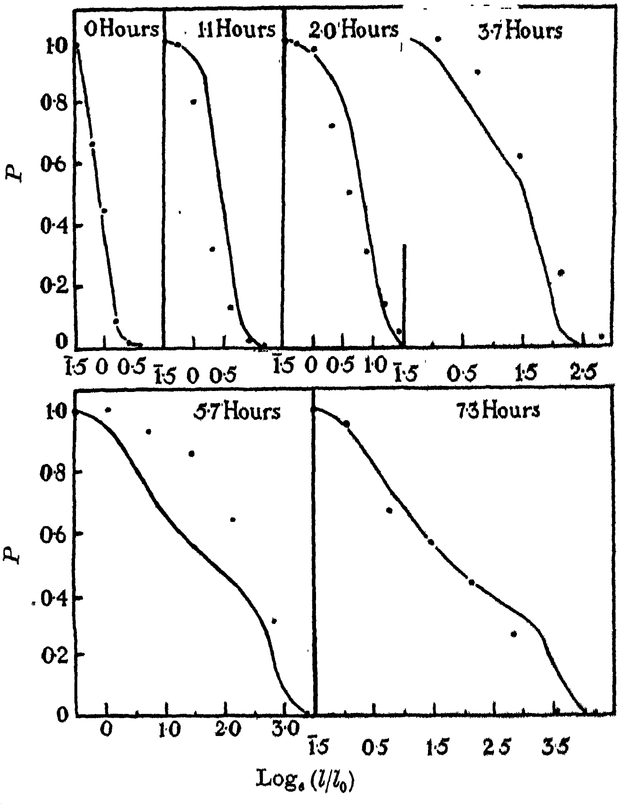

Fig. 17—Length distributions, *B. coli* irradiated in broth. Curves theoretical, points experimental.

is a phenomenon which has been established for growth in tissue cultures (Strangeways and Hopwood 1926), while the production of abnormally long forms by the action of radiation in conditions favourable to growth has been observed with a variety of biological materials (Luyet 1932; Robertson 1932).

Our thanks are due to the Medical Research Council, the British Empire Cancer Campaign, and Radium Belge (in conjunction with Messrs Watson and Sons) for facilities afforded which made the investigation possible.

SUMMARY

Experiments are described on the lethal action of γ-radiations upon aqueous suspensions of *B. coli* and spores of *B. mesentericus*. Exponential survival curves are obtained, the mean lethal ionization dosages being approximately equal to those previously obtained for β-rays under conditions in which the rate of death was very much greater.

Experiments upon *B. coli* in nutrient media are also described which suggest that the lethal action of the radiation proceeds independently of growth in the medium. Under certain conditions abnormal *B. coli* in the form of long filaments have been obtained. A quantitative analysis of the results of viable counts, total counts, and length distribution measurements leads to the conclusion that the production of long forms is due to division being inhibited, while growth, in the sense of increase of volume, is unaffected.

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Pituitary Control of Alimentary Blood Flow and Secretion

Changes in the Stomach produced by the Administration of Posterior Pituitary Extract

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[Plates 2, 3]

A preliminary report on the gastric lesion associated with the injection of a posterior lobe pituitary extract has been given (Dodds, Noble and Smith 1934; Dodds, Hills, Noble and Williams 1935). To assist in determining the mechanism of this reaction, careful histological studies have been made of the changes in the stomach mucosa.

It has been found that the gastric lesion may be produced by the subcutaneous, intravenous or intracisternal injection of any posterior pituitary extract containing the pressor fraction. Control extracts of many tissues including anterior and intermediate lobes of the pituitary have been shown to be inactive. Similarly, the oxytocic fraction of the posterior lobe is without effect. For the extract to be effective when given by mouth, it is essential that the solution be in dilute acetic acid, similar to standard B.P. pituitary extract.

The acute gastric change has been produced in the common laboratory animals including the monkey, cat, rabbit, guinea-pig, rat and mouse. These results have been confirmed by Bergami (1935) and others (Lucchesti and Zilioli 1935). Nedzel (1936) has shown that dogs also give gastric lesions. Typical chronic gastric ulcers may be produced in the rabbit

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* Leverhulme Fellow, Royal College of Physicians.

† Working with a grant from the Medical Research Council.

(Dodds and others 1935; Dodds and Noble 1934), the rat and the dog (Nedzel 1936) without difficulty.

The active substance from the posterior lobe of the pituitary has been obtained by various methods of extraction. The acetone picric acid process of Dodds and Dickens (1924) has been applied to frozen dissected ox pituitaries, and the crude extract tested. Extraction of the acetone dried gland with dilute acetic acid, similar to the method described in the *British Pharmacopoea*, has yielded standard pituitary extract B.P. More highly purified preparations have been made according to the process described by Kamm and his collaborators (1928) for the separation of the oxytocic and pressor principles.

If a rabbit is given a large subcutaneous dose of one of these active extracts (20 c.c. of B.P. extract or 100 mg. of the picrate), the animal at first appears very restless and in a short time becomes prostrate with a greatly increased respiratory rate. This passes off after 5–6 hr. For the following few days the animal refuses to eat. Diarrhoea is usually present, and the faeces may contain altered blood; but within a week the animal has returned to normal. If, however, the animal be killed in 24 hr. and the abdomen opened, a definite picture is presented. The upper end of the stomach has a mottled purplish red colour instead of the normal pearly grey, and occasionally the wall is markedly oedematous. The normal mucosa of the upper two-thirds of the stomach, cardia and fundus is replaced by an intensely engorged haemorrhagic mass of tissue, blackened from the acid in the stomach contents. This intense reaction stops sharply at the oesophageal opening and gradually fades above the incisura angularis; this sharp line of demarcation is found to correspond with the exact distribution of oxyntic cells in the rabbit. The pyloric end of the stomach and the duodenum are normal, except in those instances in which oedema is encountered; in these animals the whole stomach wall, including the pyloric portion, is greatly thickened, the bulk of the fluid lying between the muscle and mucous coats. Perforation during the acute stage of the lesion has never been observed. This typical appearance is shown in fig. 1, Plate 2.

The microscopic appearance can be best studied by taking sections at various times after administration of the extract. The immediate effect of intravenous injection can be seen with the naked eye to be an entire blanching of the stomach mucosa. One hour after subcutaneous injection, marked capillary dilatation and engorgement have occurred. This is noticed particularly in the middle zone of the mucosa which contains mainly oxyntic cells, fig. 2, Plate 2. Evidence of change is also seen in the oxyntic cells themselves, such as pyknosis of their nuclei and irregularity of the cell outline. Sections after 2 hr. show the same process which has become more extensive. The lining epithelium has been destroyed, with marked cellular necrosis, and this condition has spread down among the peptic glands: fig. 3, Plate 2. After 4–6 hr. the destructive process is general throughout the acid-bearing area of the stomach. The lining epithelium has been completely destroyed as well as most of the mucosa. The normal arrangement of cells has been replaced by necrotic tissue, extending down to, but rarely below, the muscularis mucosa. The larger blood vessels appear to be normal. Thrombosis of the larger veins is not observed, fig. 4, Plate 2.

The pyloric mucosa shows nothing abnormal, except that in one experiment out of seventy-six a rabbit showed a typical gross lesion in this area. A section of this showed an acute lesion with the presence of heterotopic oxyntic cells. Occasionally large areas of mucosa slough off and may extend into the pyloric area, but this is uncommon.

Stomachs examined at longer intervals after the administration of the extract indicate that healing and regeneration are rapid; even following very large doses, the gastric mucosa appears to differ little from the normal after 10 days. During regeneration there is some fibroblastic proliferation both in the mucosa itself and in the subcutaneous connective tissue.

Approximately 25 % of the animals develop a typical thickened punched-out ulcer; these have always been single and situated at the cardiac end near the greater curvature. Perforation occurs in some, and omentum is found patching over the ulcer, but death has not resulted from peritonitis in any of these animals, fig. 5, Plate 3.

Macroscopically the ulcers vary in size from 5 to 25 mm. in diameter, and are sharply demarcated with slightly raised thickened edges and a base of greyish granulations. On section they show the characteristics of a chronic ulcer, including fibrosis of the coats and a floor of granulation tissue in which are numerous inflammatory cells; the mucosa at the edge of the ulcer shows regeneration.

In animals other than rabbits, the acute lesion has a somewhat modified appearance. In guinea-pigs the distribution is essentially the same except that the distal line of demarcation is not so distinct. The general reaction is not so severe and the intense blackening and oedema are rarely found. In mice and rats the lesion occurs with greatest intensity immediately distal to the squamous epithelium which lines the upper half of the stomach and which remains normal. In the mildest reaction the folds of the rugae are merely engorged and bluish, whereas in the intense forms bright red haemorrhages are seen. Chronic ulcers are frequent in the rat following the administration of the extract by mouth. In the cat there are no sharp limits to the lesion, but it tends to fade near the pylorus. The reaction is typically an engorgement followed by haemorrhage and erosion of the mucosa. In the monkey the most noticeable changes are small areas of haemorrhage occurring on both greater and lesser curvatures extending down to the pylorus. Histologically the lesion in the various animals appears to be essentially similar in character to that of the rabbit, the main difference of appearance and intensity is attributable to variations in the distribution of the oxyntic cells and in the acid content of the stomach.

The injection of smaller doses of the extract may modify the extent of the gastric lesion considerably. Small doses in the rabbit produce localized areas of necrosis which appear as typical acute erosions of the mucosa.

From the distribution of the lesion it is clear that the presence of acid-secreting oxyntic cells is an essential factor in its production. The fact that it may be induced in all animals shows that the gastric lesion, unlike that caused by many other substances, is not a species peculiarity. Atropine, pilocarpine, adrenaline, and histamine, in large doses, have never given a typical pituitary lesion in the rabbit. The picture could, however, be closely imitated by introducing N HCl into an anaesthetized animal's stomach. Here the destruction of the mucosa usually continued from the fundus into the pyloric end. In some cases a mild gastric lesion, similar in all respects to the typical pituitary one, was found following the intravenous injection of barium chloride, 15 mg./kg.

It was hoped that pituitary removal would cause some definite histological changes in the stomach. The presence of acute gastric erosions following hypophysectomy has been described by other workers (Keller, D'Amour and Hare 1934). In a series of 165 rats and 65 cats, after this operation, we have found that small erosions occur more frequently than in control animals, but chronic ulcers have never been produced. These findings are of doubtful significance because of the often impaired general nutrition of the animal before death, and of the proximity of the hypothalamic connexions to the operative field for pituitary removal; damage in this area of the brain being known to give rise to gastric lesions (Beattie 1932). Some alterations in the oxyntic cells have, however, been observed; these occasionally contain minute vesicles and the granules appear finer. A "plasma cell" or cartwheel arrangement of the chromatin in the nucleus of these cells—similar to that described by Selye and his collaborators (1933) in the ovary of hypophysectomized rats—has also been noted.

CONCLUSION

A description of the macroscopic and microscopic changes in the pituitary lesion of the stomach has been given.

A similar lesion has been produced only by the injection of a barium salt.

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DESCRIPTION OF PLATES 2, 3

FIG. 1—Distribution of lesion in rabbit's stomach.

FIG. 2—Rabbit's stomach 1 hr. after injection of pituitary extract (× 60).

FIG. 3—Rabbit's stomach 3 hr. after injection of pituitary extract (× 60).

FIG. 4—Rabbit's stomach 6 hr. after injection of pituitary extract (× 60).

FIG. 5—Perforated chronic gastric ulcer in a rabbit. The acute reaction was produced by an injection a few hours before the animal was killed.

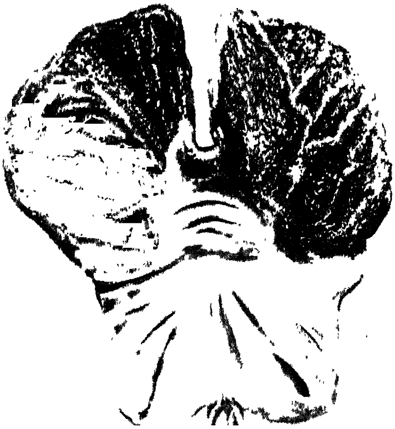

FIG. 1

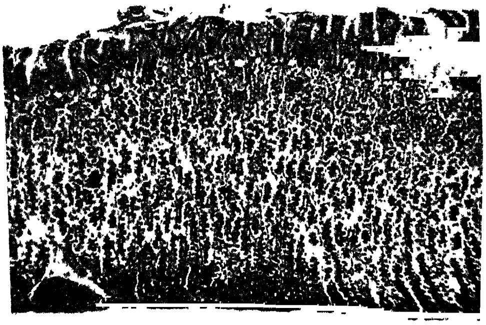

FIG. 2

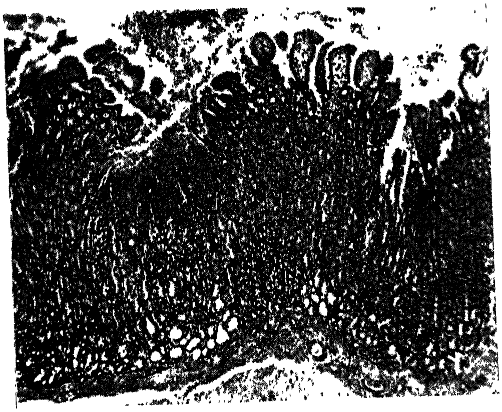

FIG. 3

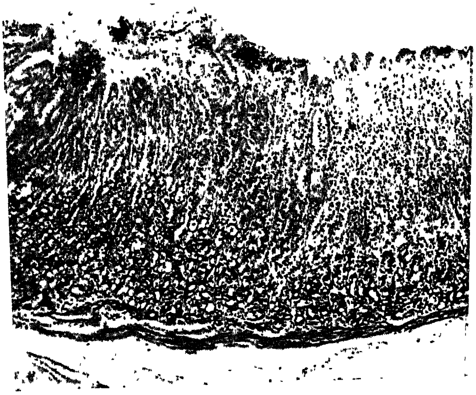

FIG. 4

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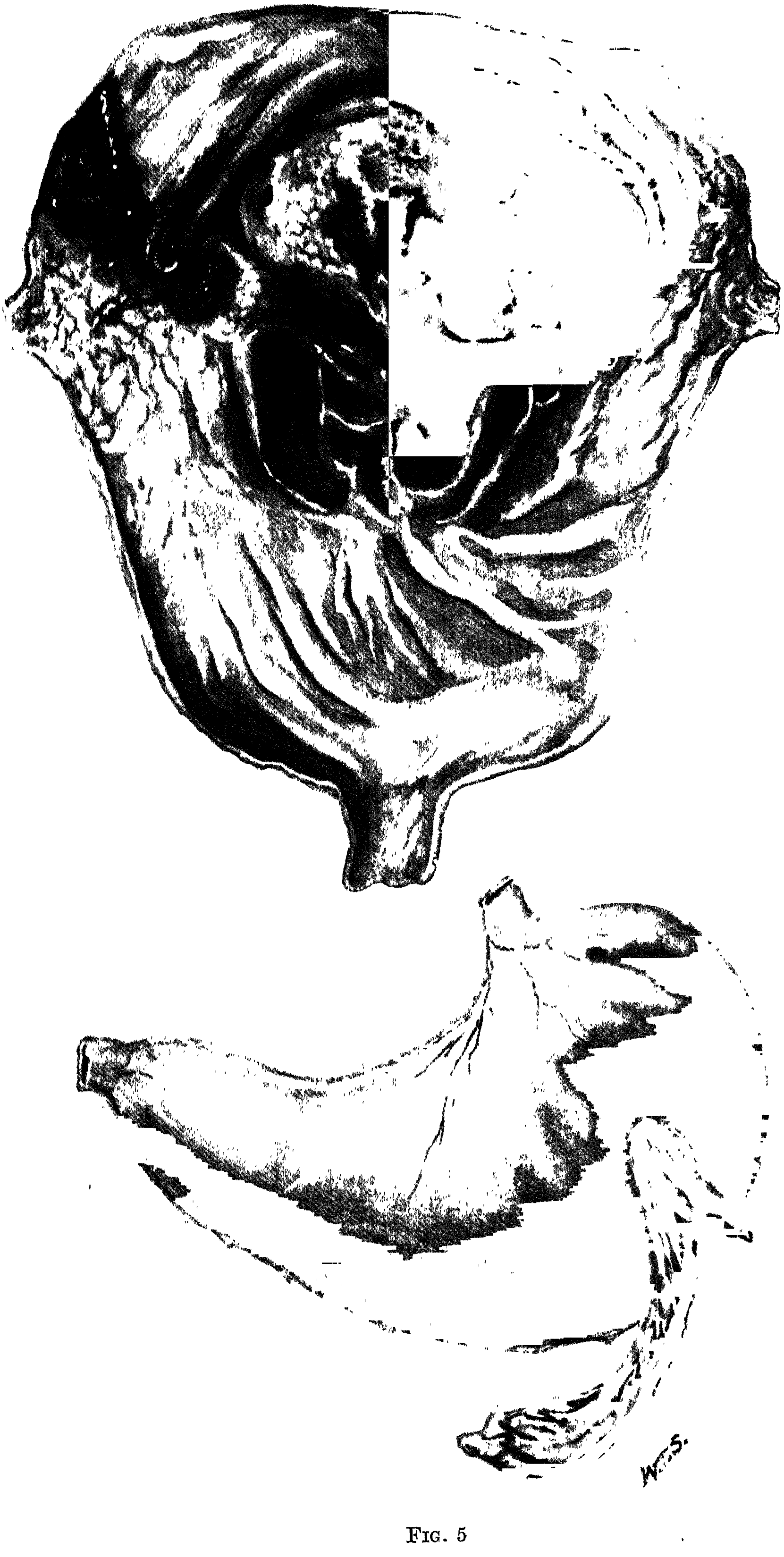

Fig. 5

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Pituitary Control of Alimentary Blood Flow and Secretion

The Effect of Posterior Pituitary Extract on the Alimentary Secretions of Intact Animals

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The essential relationship between the gastric lesion produced by pituitary extract and the oxyntic cell-bearing area of the stomach mucosa has been pointed out in the previous paper. This and the fact that animals with gastric contents of high acidity are the most susceptible, together with the demonstration that neutralization of this acidity will prevent the production of a lesion (Dodds, Hills, Noble and Williams 1935), suggested that acid secretion by the stomach played an important part in the production of the lesion. It was necessary, therefore, to continue these observations on gastric secretion.

In the following experiments on gastric secretion a standard routine had been adopted and has been employed except where otherwise stated.

In acute experiments rabbits have been anaesthetized with sodium amytal and cats with "Nembutal". A glass gastric cannula has been stitched into the most dependent part of the stomach, and the pylorus tied off. Animals with a previously established permanent fistula have not had the pylorus ligated. The cats have been kept without food for 24 hours previous to any tests, but in the case of the rabbits the stomach was emptied by a technique previously described (Dodds and others 1935). A 30 min. specimen of fasting gastric juice had been collected, and in most cases this included the fasting contents of the stomach. Ergamine acid phosphate (histamine) has been administered by subcutaneous injection, and halfhourly specimens of juice obtained. Titration for acidity was performed with 0·05 N sodium hydroxide with Toepfer's reagent and phenolphthalein as indicators.

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So far as possible the results have been tabulated on graphs and the dose of substances indicated. A mean curve of secretion, together with all the individual acid values has been given; the maximum deviation being recorded as a shaded area on the graph. Curves for the average volume of juice for each 30 min. period have also been given.

In certain cases the tests have been performed on cats without anaesthetic. In these animals a permanent gastric fistula has been established at least 2 weeks previously under aseptic conditions. These cats have been carefully trained so that they submitted to the tests without the slightest resentment. The secretion was collected in a rubber balloon fixed by a cork into the fistula. The animals were allowed to walk about at will and did not attempt to interfere with the receptacle or the taking of specimens.

HISTAMINE STIMULUS

Rabbits—The normal response to histamine has been obtained in twelve normal rabbits. The results of these tests are given in fig. 1. The marked stimulation of acid and the volume of juice is clearly seen.

The effect of an injection of pituitary extract has been examined by observing the way in which it modifies the normal histamine response and the excretion of neutral red in the stomach juice. If a normal rabbit be injected with 1 c.c. of a 1 % solution of neutral red intramuscularly at the same time as the histamine is given, the dye appears in the gastric juice in the first half-hour specimen. In fig. 2 the appearance of the dye is marked with an arrow; 2 c.c. of B.P. pituitary extract per kilo was given intravenously 30 min. before histamine, and the acid and volume curves of four rabbits are recorded.

In a previous report (Dodds, Noble and Smith 1934) it was shown that acetone picric acid process extracts of control tissues did not produce any effect on the stomach mucosa. Control tissue extracts were found to have no effect on histamine induced secretion, whereas posterior pituitary extracts similarly prepared inhibited this secretion. Oxytocin was also found to have no inhibitory action.

These results show that pituitary extract greatly diminishes or abolishes the profuse secretion of acid juice normally produced by histamine. Control tissue extracts have no effect and the action of oxytocin is no greater than might be expected from the small fraction of vasopressin it contains.

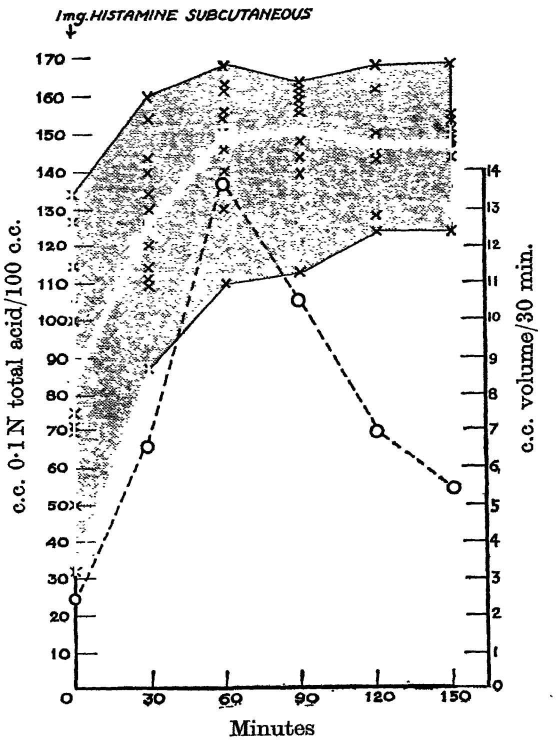

FIG. 1—Gastric secretion in rabbits. Histamine stimulus.

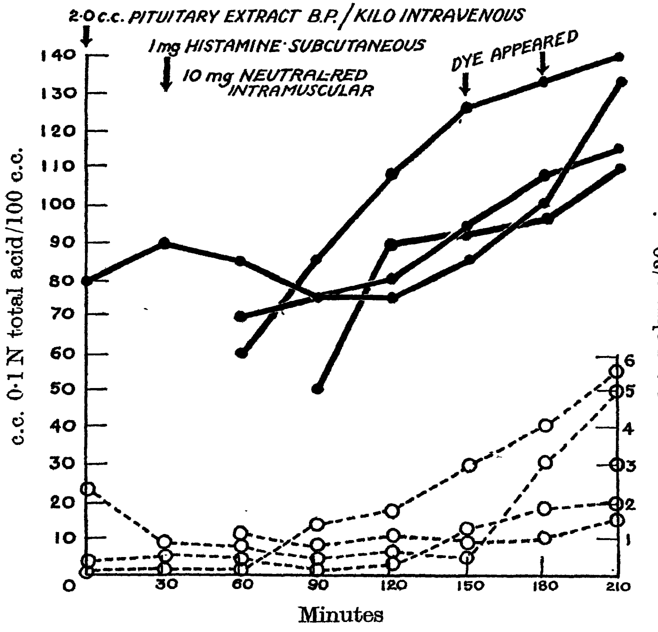

FIG. 2—Inhibition of gastric secretion in rabbits. Histamine stimulus.

—●— c.c. 0·1 N total acid/100 c.c. ----○---- c.c. volume/30 min.

Although it is difficult to estimate acidity accurately because of the small volume of juice, it would appear that the most dramatic action of the vasopressor fraction of pituitary extracts is to inhibit the volume of juice secreted.

Cats—The unusual type of digestion associated with the herbivorous diet of the rabbit and the resulting difficulties in gastric research, suggested that experiments should be repeated in animals having a digestive system more closely related to that of the human. For this reason most of the results to be described have been obtained using cats as the experimental animal.

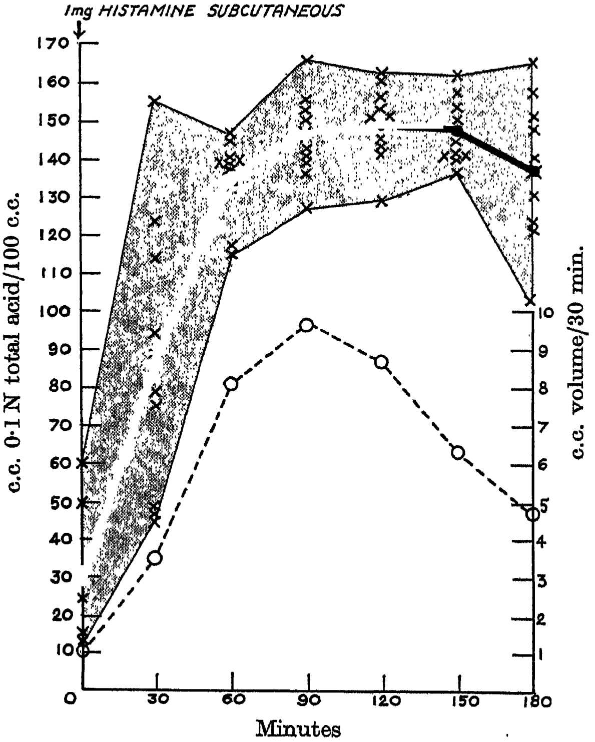

FIG. 3—Gastric secretion in anaesthetized cats. Histamine stimulus.
—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

The response to histamine has been found to be within normal limits for animals with an acutely established gastric fistula and for those with a permanent fistula. The results, therefore, of ten tests in nine different cats are given together in fig. 3. In this series all the animals were anaesthetized throughout the test. A more recent series of animals, however, have been tested without any anaesthetic. The curves for these experiments are given in fig. 4. The increase in volume is seen to be so marked and the falling off of acidity so much more rapid as compared with anaesthetized cats, that these results have had to be tabulated separately.

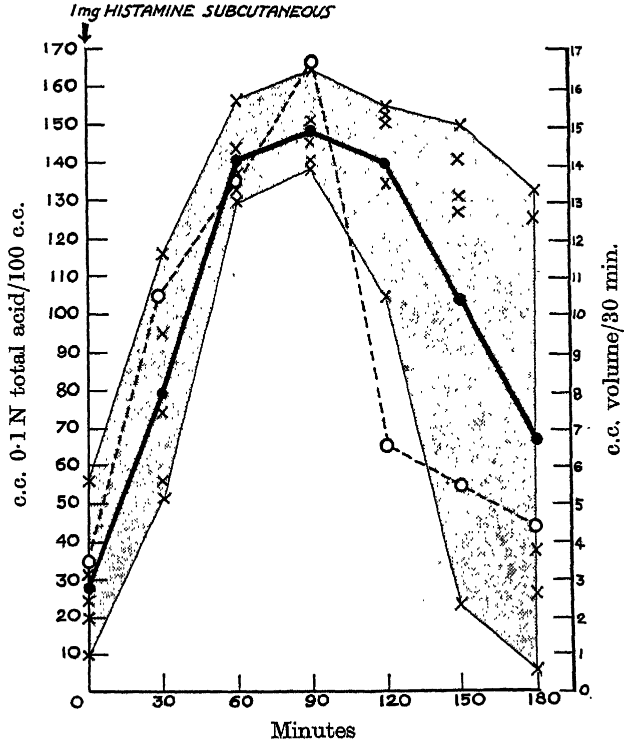

FIG. 4—Gastric secretion in unanaesthetized cats. Histamine stimulus.

—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

In fig. 5 the effect of giving pituitary extract B.P. in doses from 0·25 to 5·0 c.c. intravenously 60 min. after the secretion has been stimulated by histamine is shown. Again, as in the rabbit, the inhibiting action appears to be most marked on the volume of gastric juice. Following a dose of 0·25 c.c., secretion recommenced in about 1 hr.

INSULIN STIMULUS

The action of insulin on gastric secretion has been reported by a number of workers. Although some (Ivy and Fisher 1924; Collazo and Dobreff

1924) have stated that insulin produced no effect on secretion, others have demonstrated a definite stimulation (Okada and others 1929; Boldyreff and Stewart 1932; La Barre and De Cespédès 1931; Roholm 1930). The effect is believed to be caused by a lowering of the blood sugar, and the action would appear to be a nervous stimulation through the vagus.

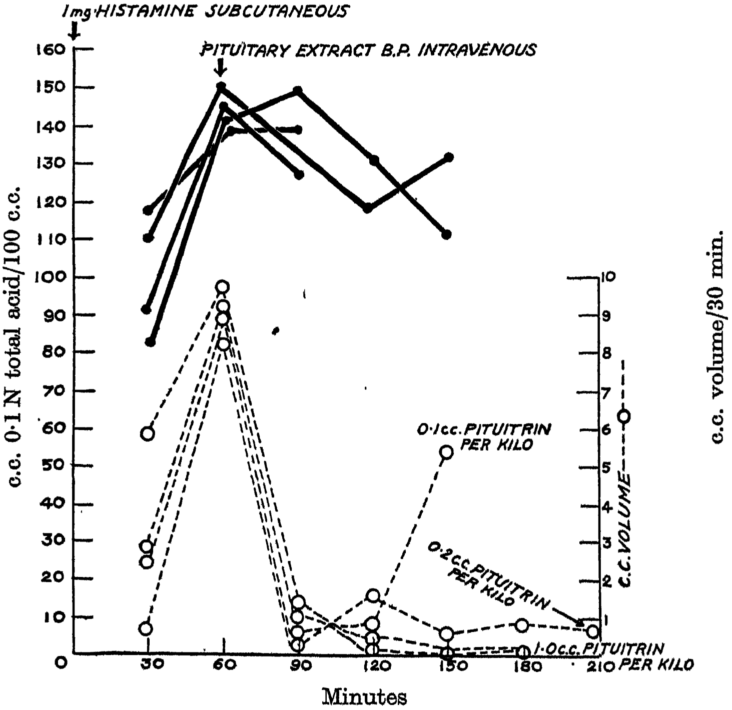

Fig. 5—Inhibition of gastric secretion in cat. Histamine stimulus.
—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

We have studied a series of cats having permanent gastric fistulae, using 2 units of insulin (subcutaneous injection) as a means of stimulating gastric secretion. Commercial and crystalline insulin have been tested. The animals used have been non-anaesthetized since all barbiturate anaesthetics and chloralose abolish the insulin effect. Fig 6 gives the results of fourteen tests on eleven different animals. The marked stimulation in acid is apparent, commencing 30–60 min. after the injection of the insulin. The pepsin values, expressed as Fuld units, are given in Table I, and are surprisingly high. It would seem that insulin is the most powerful stimulant for pepsin secretion in the cat. Also, as insulin stimulates all the component

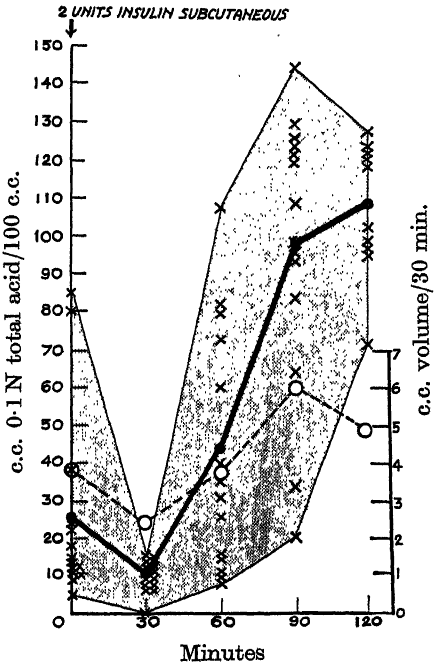

FIG. 6—Gastric secretion in cats. Insulin stimulus.
—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

TABLE I—PEPSIN IN GASTRIC JUICE AFTER INSULIN (EXPRESSED IN FULD UNITS)

Fasting contents	After insulin			
	30 min.	60 min.	90 min.	120 min.
4	—	256	512	512
<4	—	128	1024	1024
<4	<4	32	512	512
32	<4	4	—	1024
64	4	1024	1024	1024
64	<4	512	512	512
<4	<4	1024	512	—
64	—	512	512	—
<4	16	—	1024	1024
—	—	2048	1024	—
—	—	—	1024	1024
—	—	—	512	512
—	<4	—	512	1024

parts of gastric juice, it evokes a much greater physiological response than does histamine.

The inhibiting action of pituitary extract has been demonstrated by giving varied doses to a cat (fig. 7). The animal received five injections over a 2-day period before the test, the total dose being indicated on the graph. A rest of at least 1 week between each test was allowed. For comparison a normal curve on the same animal is given. The action of the equivalent of 0·01 c.c. of B.P. pituitary extract is slight, whereas 4 c.c. completely abolishes any stimulatory effect of the insulin. Control experiments have shown that the antagonistic action on blood sugar between pituitary extract and insulin is not responsible for the inhibition of gastric secretion.

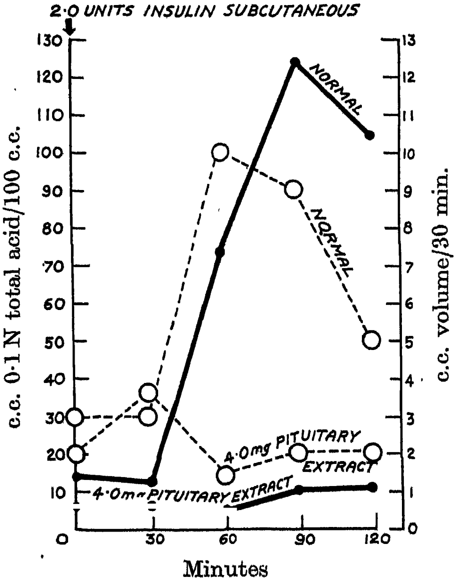

Fig. 7—Inhibition of gastric secretion in cat. Insulin stimulus.

—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

Other Stimuli

A normal physiological stimulus to gastric secretion is that produced by the taking of food. This method has been employed on cats with a permanent fistula. The animals were given 50 g. of ground meat to eat. Immediately after this the stomach was washed clean with warm water through a catheter inserted through the fistula. Five minutes was then allowed for any water to drain out before commencing to collect the specimens. Owing to the short duration of the flow of juice obtained by this means samples were taken every 15 min. The inhibiting action of pituitary extract B.P. can be seen from fig. 8. A total of 5 c.c. was injected subcutaneously to this animal over a 2-day period; its normal response is also included in the graph.

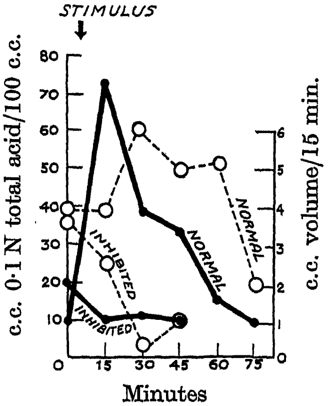

FIG. 8—Inhibition of gastric secretion in cat. Sham feeding stimulus.

—•— c.c. 0·1 N total acid/100 c.c.
----o---- c.c. volume/15 min.

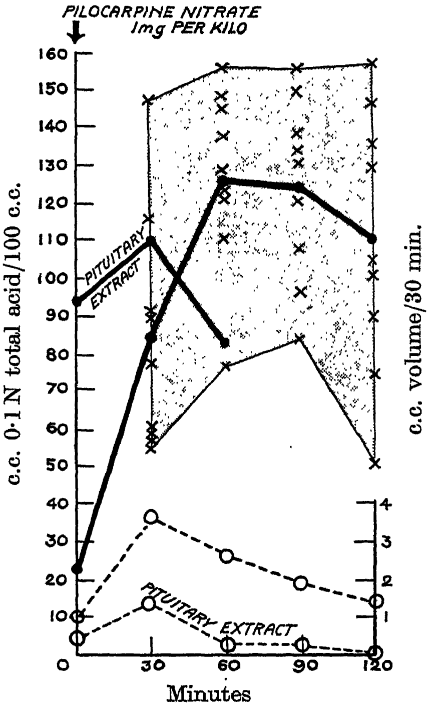

FIG. 9—Inhibition of gastric secretion in cats. Pilocarpine stimulus.

—•— c.c. 0·1 N total acid/100 c.c.
----o---- c.c. volume/30 min.

Intravenous pilocarpine in the dose of 1 mg./kg. has been used as another form of gastric stimulus on anaesthetized animals. The response of nine cats and the inhibition produced when 1 c.c. of pituitary extract B.P. was given intravenously a few moments after the pilocarpine is shown in fig. 9.

The dramatic action of pituitary extracts on the stomach suggested that a similar action might occur on pancreatic function. In these acute experiments fasted cats were anaesthetized with "Nembutal", and the pylorus ligated. A small glass cannula was fixed into the pancreatic duct for the collection of juice. Intravenous injection of secretin was used as a means of stimulation. A preliminary dose of 1 mg. was given and the normal immediate response elicited. Specimens were measured every 15 min. Following a 1 hr. rest after secretion had stopped, the cat was given intravenous pituitary extract B.P. 5 c.c. followed by the control dose of secretin. From Table II it is seen that the secretion of pancreatic juice is abolished in three experiments of this type. In one animal the secretin was allowed to start secretion which immediately ceased after the pituitary administration.

TABLE II—EFFECT OF B.P. PITUITARY EXTRACT ON PANCREATIC SECRETION

	Total juice secreted		
Stimulus	Cat 138 c.c.	Cat 159 c.c.	Cat 160 c.c.
Secretin	1·7	—	—
Secretin	0·86	0·45	1·48
Pituitary extract + secretin	0·00	0·02	0·03

The effect of pituitary extract on salivary secretion has not been recorded, but while observations were being made on the gastric effect of pilocarpine on cats, it was noted that at least temporary diminution of the flow of saliva occurred after the injection of pituitary extract.

The known vascular action of vasopressin, and the fact that barium salts produced a similar gastric lesion, suggested that a vasoconstriction was probably related to the diminished secretion. The effect of intravenous barium administration following histamine was therefore tested on gastric secretion of both the rabbit and the cat. The results were found to be similar in both species and the action on the cat is seen in fig. 10. β-phenylisopropylamine, another substance producing vasoconstriction, also produced a similar, though less striking, diminution of gastric secretion in cats.

DISCUSSION

The nervous, humoral and intestinal types of stimuli are well recognized as those causing gastric secretion in a normal animal. Of all substances which initiate the secretion, histamine is the most powerful, and the marked increase in the volume of juice and in acidity can clearly be seen from the graphs. The injection of relatively small doses of pituitary extract, however, is followed by a complete cessation of secretion of gastric juice.

The definite stimulating action of insulin on gastric secretion has been confirmed. This action, although not so powerful as that produced by histamine, gives rise to a juice of more normal composition, the enzyme content being very high. Secretion produced by sham feeding or pilocarpine is of a less profuse nature. These various stimuli represent all types which may take place in a normal animal except, possibly, that caused by intestinal action. In all cases it is readily demonstrable that pituitary extract abolishes the stimulatory effect and may produce a condition of achlorhydria in the animal.

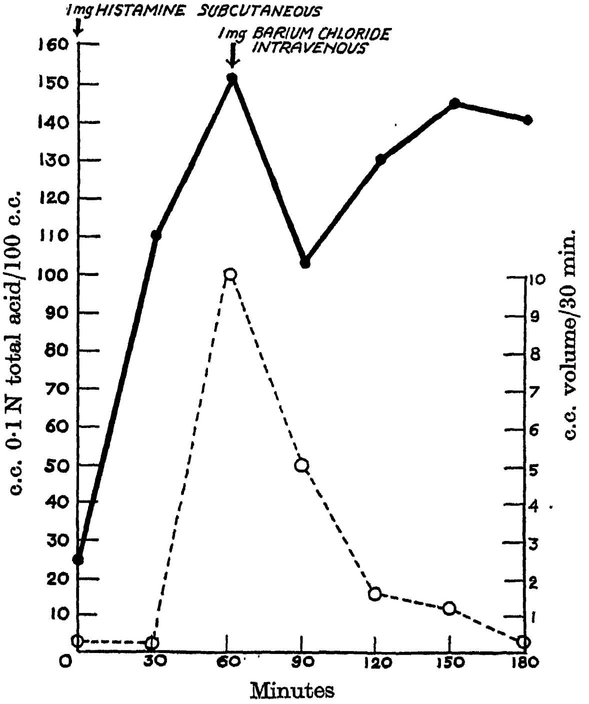

FIG. 10—Effect of barium chloride on gastric secretion in cat.

—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

That such a reaction is not specific for the stomach is seen from the fact that pancreatic secretion is similarly abolished. Other substances capable of producing vascular constriction may cause an effect similar to pituitary extract. This fact, and the finding that the oxytocic fraction of these extracts has no effect, suggests that the actions described are due to the vasopressor fraction. It would appear from these observations that an essential vascular factor must have an intimate relationship to glandular secretion.

The formation of the gastric lesion is obviously closely connected with the cessation of secretion. The necessity for an acid medium in the stomach contents in the production of a lesion has been shown by the following observations: histological studies indicate that the localization of the reaction is confined to areas which contain acid-secreting cells. The stomach mucosa remains normal after the pituitary injection when the gastric juice is neutralized in the rabbit. The animals most susceptible are those in which the stomach contents are continuously acid. The importance of a mechanical factor in the production and localization of gastric lesions has been stressed by McCann (1926). It is possible that the distension caused by food in the rabbit's stomach may produce a more extensive exposure of the mucosa to acid and thus enhance the lesion. Difficulty is experienced in producing an effect in the cat where a thick protective layer of mucus covers the lining of the stomach. It is seen, therefore, that in an animal where the acid of the stomach has direct access to the gastric mucosa, if all secretion be suddenly stopped, necrosis of the mucosa results.

It would appear from these observations that a normal continuous secretion is essential to prevent auto-digestion of the organ. As it has been shown that a vascular factor is closely related to the maintenance of normal secretion, it follows that the integrity of the stomach mucosa is also related to this factor.

Conclusions

It has been demonstrated that the stimulating effect on gastric secretion, produced by a number of different types of stimuli, is abolished by the action of the vasopressor fraction of posterior lobe pituitary extracts. The volume of juice is primarily affected.

Pancreatic stimulation due to secretin has been shown to be similarly inhibited by pituitary extract.

Other vasoconstrictors have been found to produce an effect similar to pituitary extract on gastric secretion.

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Pituitary Control of Alimentary Blood Flow and Secretion

The Effect of Alterations in Blood Flow on Gastric Secretion

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In the preceding article it was shown that some substances which act on the blood vessels exert a profound effect on gastric secretion. To investigate this action more thoroughly experiments have been devised to show the normal vascular response of the stomach to stimuli, and the effect on this response of substances which act on gastric secretion.

METHOD

The following procedure has been adopted in preparing the animal for the measurement of blood flow through the stomach.

A fasted cat was anaesthetized with "Nembutal", the external jugular vein cannulated, and a blood-pressure cannula inserted into the carotid or femoral artery. The abdominal contents were then exposed by a long mid-line incision. The vascular supply to the spleen was tied off in a series of ligatures close to that organ. The spleen was then removed. A glass fistula was stitched into the posterior surface of the fundus of the stomach, and brought out to the back through a grid-iron incision in the subcostal region. The pylorus was tied. The animal was now eviscerated from the rectum to duodenum and the superior mesenteric vein dissected out. A cannula was tied into the vein and led to a manometer to be described later. The peripheral end of the mesentery was tied off in a single ligature.

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Twenty-five mg./kg. of heparin was given at this stage as an anti-coagulant. The external jugular vein was now connected to a warmed condenser through which blood was transfused back into the cat. The blood pressure was recorded in the usual manner. The means by which the blood flow from the stomach was collected may be seen from the accompanying drawing (fig. 1). The normal pressure exerted by the liver was measured by tube *A* of the manometer. The top of tube *B* from which the blood was collected was adjusted to the same level as in tube *A* which was then clamped. If a weak clamp were now put on the portal vein *C*, the blood from the stomach was forced back into the mesenteric vein and out into a graduated container. The flow for a given time was measured, the portal clamp removed, and the blood immediately transfused back into the animal.

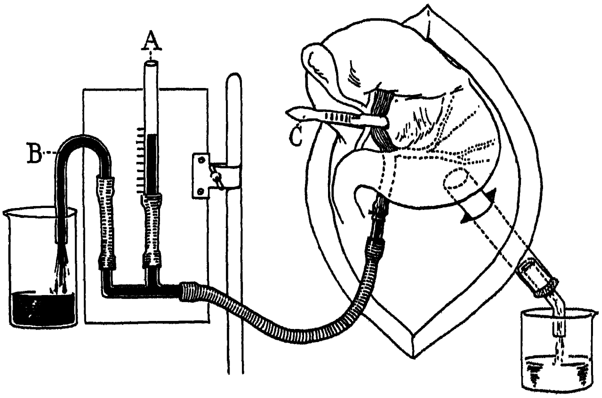

FIG. 1—Diagram of method of measuring blood flow through stomach.

To prevent any obstruction in the manometer, the tube was washed through with saline after each reading. Gastric juice specimens were collected every 15 min.

In early experiments of this type no allowance was made for pressure variations in the liver, and the blood was collected continuously from the mesenteric vein; records were made on a Condon drop recorder. With this method the continuous transfusion necessary caused fluctuations in blood pressure which influenced the rate of flow to the stomach. A modification was used for all later experiments. In this the portal vein was clamped for only 30 sec. every 15 min. in order to measure the blood flow. The blood was immediately transfused so that for 12–13 min. out of every 15 the circulation to the stomach was completely normal. Under these conditions the stomach produced a secretion comparable to controls when stimulated with histamine. By collecting the blood from the stomach against a hydrostatic pressure equal to the venous pressure in the portal vein, the blood flow through the stomach was measured under conditions as normal as possible.

RESPONSE TO HISTAMINE

At the start of these experiments the blood pressure ranges from 130 to 170 mm. Hg. but shows a gradual decline throughout. The blood flow through the stomach at first is usually very high—15 to 30 c.c./min.—but falls off, at first rapidly, and then more gradually, until a lower and more constant rate is reached. Gastric secretion is found to diminish quickly as the blood flow decreases.

The effect of stimulating gastric secretion by histamine injections was investigated in eleven normal cats, the drug being administered after the preliminary fall in blood flow.

The eight experiments performed, using the modified method as described, are shown in Table I.

TABLE I

		Blood flow as c.c. per minute							
Time min.	Stimulus	Cat 120 B	Cat 121 B	Cat 181	Cat 183	Cat 184	Cat 185	Cat 186	Cat 189
0	—	—	—	—	—	24	—	17	—
15	—	18·4	5·4	11	30	12	11	9	24
30	—	12·0	4·0	8	16	14	7	11	19
45	1 mg. histamine	10·8	3·7	7	15	11	8	13	15
60	—	8·6	4·6	9	22	11	9	9	10
75	—	9·2	5·2	8	19	12	9	9	8
90	—	12·0	7·2	9	17	14	8	9	7
105	—	12·1	8·4	9	—	17	7	9	8
120	—	—	11·0	9	—	16	—	10	8
135	—	—	10·0	8	—	13	—	11	10

These results show that the injection of histamine caused a definite rise in the blood flow. In some cases the initial fall in blood flow continued for some time after the histamine injection, but then showed a rise. In four cases where the increase in blood flow was the most marked, a secretory curve was obtained comparable to a control under these conditions. The best example is given in fig. 2 where the curves showing the increase in volume and acidity of the gastric secretion are seen to be parallel to that of the blood flow. In some cases the secretory response has been small—owing, no doubt to the effect of the extensive operative technique necessary for the preparation of the animal, but a marked secretion has never been obtained in the absence of an increased blood flow. Other workers (Lim, Necheles and Ni 1927) have failed to demonstrate consistently an increase in blood flow accompanying secretion. They suggest that such a response might normally occur but not under the very artificial conditions of their experiments with the vivi-perfused stomach. From the results which we have obtained it is obvious that during active secretion of the stomach simultaneous increase in blood flow normally occurs. The greater the secretion which is formed, indicating the more normal condition of the animal, the greater is the vascular response.

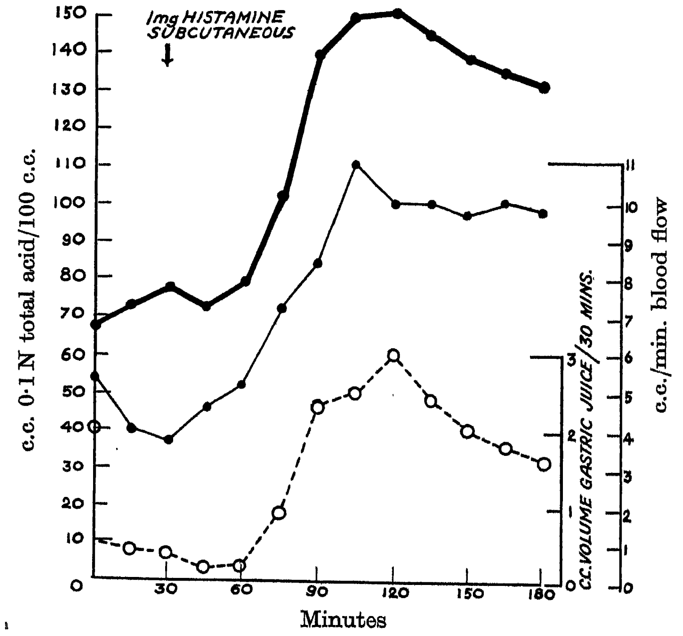

Fig. 2—Normal gastric and blood flow response to histamine.

—●— c.c. 0·1 N total acid/100 c.c. —●— c.c./min. blood flow
----○---- c.c. volume gastric juice/30 min.

Pilocarpine Stimulus

As all the preceding results were obtained as a response to histamine, it was necessary to see if a similar effect would be produced by another type of stimulus. Three cats were therefore tested by the same method with 1·75 mg. pilocarpine nitrate given intravenously. The results from one of these are recorded in fig. 3. They show the same type of parallel curves as do the histamine experiments, the rise in blood flow accompanying the rise in acid and volume of gastric juice. The other animals failed to secrete acid and the blood flow fell off throughout.

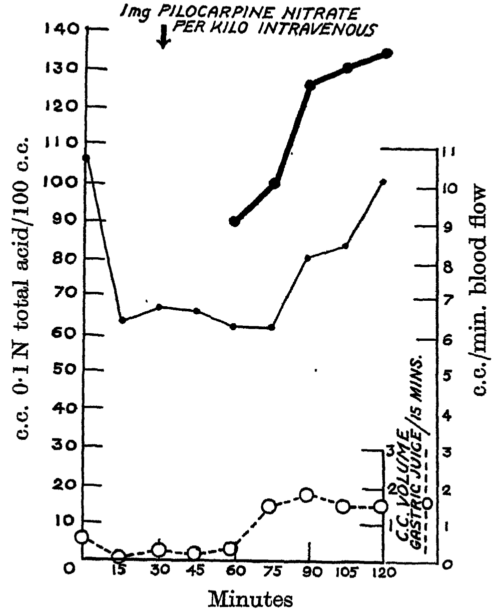

Fig. 3—Normal gastric and blood flow response to pilocarpine nitrate.

—•— c.c. 0·1 N total acid/100 c.c. —•— c.c./min. blood flow
----o---- c.c. volume gastric juice/15 min.

Effect of Pituitary Extract

The inhibiting action of pituitary extract in gastric secretion has been previously demonstrated in the intact animal. Using the method described, the effect of B.P. pituitary extract on the blood flow has been determined. It was found that 1·5 c.c. intravenously reduced the blood flow to the stomach to about half the normal. The simultaneous records of blood flow and gastric secretion on one of these cats is given in fig. 4. Here it is seen that pituitary extract not only markedly inhibits the volume of gastric secretion, but also that the blood flow is greatly reduced at the same time. The curves for blood flow and volume of gastric juice run parallel.

Effect of Cold

From the foregoing experiments it was observed that, during the decreased blood flow resulting from pituitary extract injection, gastric secretion was inhibited. Therefore it was decided to try to simulate pituitary action by some other vaso-constrictor, preferably physical. As a result, experiments on cooling the stomach were performed, using the Elliot applicator apparatus. This apparatus circulated water, the temperature and pressure of which could be controlled, through various shaped rubber applicators.

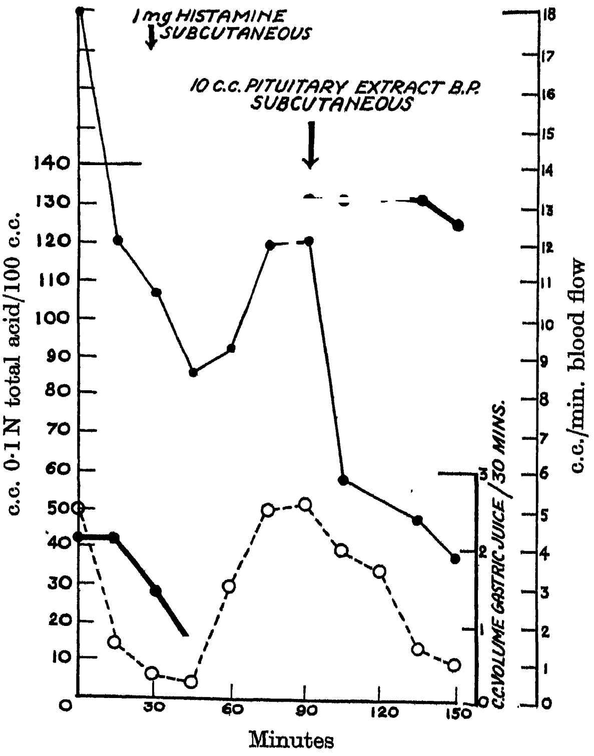

FIG. 4—Action of pituitary extract on blood flow and secretion after histamine stimulation.

—•— c.c. 0·1 N total acid/100 c.c. —•— c.c./min. blood flow
----o---- c.c. volume gastric juice/30 min.

A suitably shaped applicator was tied into the stomach and a fistula also established. The pylorus was tied and cold water circulated. The temperature of the water entering the applicator varied between 8 and 12°C. It was found that when cold water was circulating in the stomach applicator, no secretory response to histamine could be obtained. If, however, after an hour of inhibition, the water was changed to warm, gastric secretion commenced. The results of an experiment of this nature may be seen in fig. 5. The action of cold water on the blood flow through the stomach is shown in Table II, a definite decrease taking place when the water is turned on.

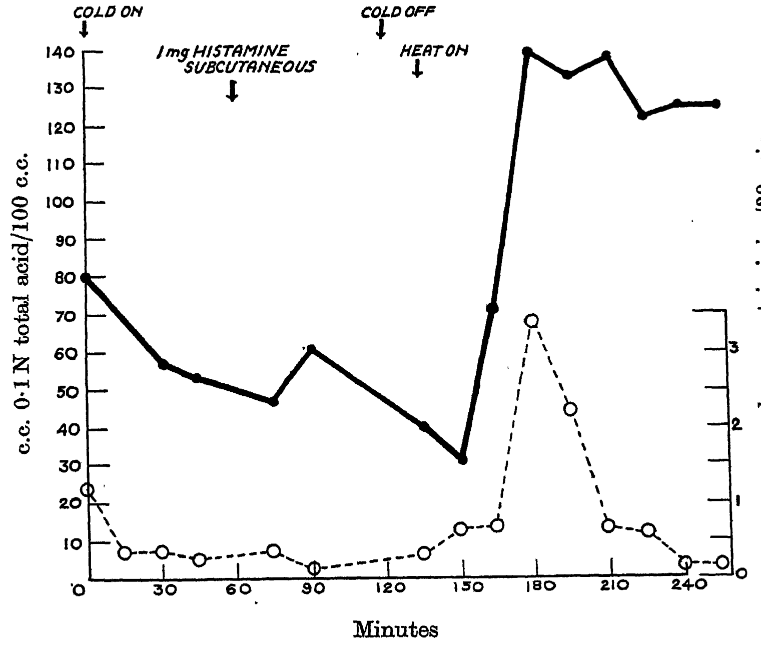

FIG. 5—Effect of vaso-constriction produced by cold on histamine secretion.

—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume gastric juice/30 min.

TABLE II

Time min.	Cat 196 Stimulus	 Blood flow c.c./min.
15	1 mg. histamine	13·0
30	—	10·0
45	—	11·0
60	—	11·0
75	—	16·0
80	Cold 12° C. on	—
95	„ „	7·0
110	„ „	7·0

EFFECT OF VASO-DILATORS

Having shown that vaso-constriction prevented gastric secretion and that the stimulating effect of histamine was accompanied by a vaso-dilatation, it was decided to see whether vaso-dilatation alone had any secretagogue activity.

ULTRA-SHORT-WAVE RADIATION

The application of heat as a means of producing vaso-dilatation was tried. The stomach was heated by means of ultra-short-wave irradiation. The applicators were placed above and below the cat so that the heating effect was concentrated on the stomach. The temperature was recorded by an alcohol thermometer. Two cats had blood flow and gastric tests done on them with this treatment, and although a large increase in flow occurred in cat 139, and a significant increase in cat 141, there was no secretion in either case (Table III).

TABLE III

	Ultra-short-wave radiation			Elliot apparatus		Erythrol tetranitrate	
Time min.	Stimulus	Cat 141 Flow, c.c./min.	Cat 139 Flow, c.c./min.	Stimulus	Cat 152 Flow, c.c./min.	Stimulus	Cat 155 Flow, c.c./min.
0	—	15·9	15·4	—	18·0	—	8·8
15	—	14·4	9·8	—	15·8	—	5·7
30	Heat on	9·6	6·6	Heat on	15·4	1 g. intra-muscularly	3·8
45	„	8·4	6·6	„	6·2	—	3·0
60	„	14·4	4·8	„	8·1	—	3·8
75	„	10·0	—	„	10·6	1 g. intra-venously	3·9
90	„	12·2	8·4	—	—	—	4·9
105	„	7·8	6·4	—	—	—	7·1
120	—	—	—	—	—	—	5·1

ELLIOT APPLICATOR

By using this apparatus and gradually heating the stomach between 100 and 120°F., an increase in blood flow was obtained, but no secretion resulted (see Table III). Experiments on intact animals failed to demonstrate any secretion of gastric juice following a gradual warming of the stomach.

ERYTHROL TETRANITRATE

Two experiments were done with this vasodilator substance injected intravenously; one with simultaneous blood flow and gastric secretion records; the other with an acute gastric fistula cat otherwise quite normal. The blood flow showed a definite increase, but in neither case was there any secretion of gastric juice.

DISCUSSION

That activity in a gland is usually associated with an increase in blood flow has been demonstrated by Barcroft and Kato (1916) on the salivary gland; and on the pancreas by May (1904). Previous efforts to study blood flow on the stomach have not been very successful because of the unphysiological conditions of the experiments, but it is of interest to note that Beaumont (1833) in his classical observations on Alex St Martin, when viewing the interior of the stomach states—"On the application of aliment, the action of the vessels is increased; the colour brightened; and the vermicular motions excited."

From results that have been described it is observed that an increase in blood flow to the stomach is a normal accompaniment of secretory activity. By producing a diminution of the blood flow during secretion it is found that the volume of juice falls off correspondingly. An increase of blood flow itself is not adequate to initiate gastric secretion, and a specific stimulus is necessary. The ease with which the secretion may be stopped after even the most powerful stimulus, by simply lowering the blood flow, demonstrates that an adequate blood flow to the organ is an exceedingly important factor.

Slight alterations in secretory activity of the stomach when the animal is subjected to alterations of body temperature have been described. The increased secretion in response to cold has been suggested by Lim, Ivy and McCarthy (1925) as being associated with splanchnic dilatation consequent on the cutaneous vaso-constriction. The experiments described on cooling the stomach demonstrate how essential an increased blood flow is for secretion.

To produce normal gastric secretion in an animal it is necessary to have a gastric stimulus, but also the condition of the vessels must be such that an adequate increase in blood flow can occur. Any factor which may alter this response in blood flow will markedly affect secretion, and in extreme cases even achlorhydria may result. The importance of this from a clinical standpoint is apparent.

CONCLUSION

A method which enables simultaneous measurement of the blood flow and gastric secretion has been described.

Following a gastric stimulus an increase in secretion and blood flow is observed. An increase in blood flow alone will not induce gastric secretion; nor will a stimulus such as histamine unless an increase in blood flow can occur.

Gastric secretion is markedly altered or abolished by any factor which prevents an adequate increase in blood flow to the stomach.

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Pituitary Control of Alimentary Blood Flow and Secretion

Gastric Secretion and Blood Flow in Hypophysectomized Animals

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(*Communicated by Sir Henry Hallett Dale, F.R.S.—*
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The effects of posterior lobe pituitary extract on the secretion and blood flow of the stomach have been demonstrated in the previous papers. As other substances capable of producing a vasoconstriction had a similar action, it was essential to establish whether the pituitary gland had any functional role in the intact animal which influenced secretion and blood flow. From the research of Krogh (1929) it would appear that in frogs the pituitary maintains a normal control over the capillaries.

GASTRIC SECRETION

For these experiments tests have been conducted on cats having a permanent gastric fistula—these being comparable to the response of normal animals as previously shown. In most cases the normal reaction of these animals was first determined. Hypophysectomy was then performed by the buccal route under aseptic conditions; ether or "Nembutal" being used for the anaesthetic. Following the operation the animals lived in a room kept at a constant temperature of 78°F. Control experiments have shown that this temperature did not appreciably alter secretion produced by the stimuli employed. Post-mortem examination of these animals included careful inspection and sectioning of the endocrine organs and sella—in many cases serial sections of the pituitary fossa and overlying brain were made.

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HISTAMINE STIMULUS

In the first experiments the response to histamine was repeated a number of times on two animals following hypophysectomy, under Nembutal anaesthesia. The results, including the response before hypophysectomy, of one cat are given in fig. 1. Six of the tests were performed from 3 to 8 weeks following the operation. One was performed 6 months later.

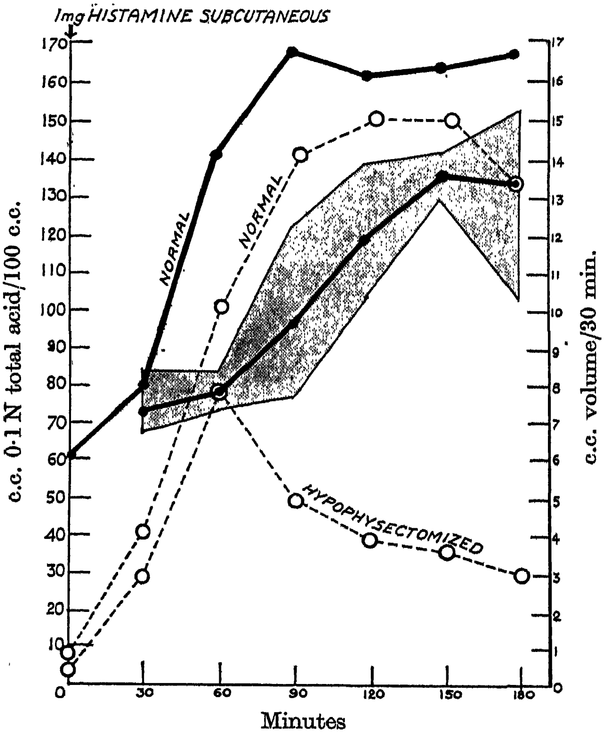

FIG. 1—Gastric secretion in hypophysectomized anaesthetized cats. Histamine stimulus.

—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

Fig. 2 shows the results of six tests on two other hypophysectomized cats and also their normal response. In these experiments the animals were not anaesthetized. These animals were used at a shorter time following hypophysectomy: 5 days to 5 weeks post-operative. A control series of repeated histamine tests over a long period on a normal cat has shown that the variation in response obtained is negligible.

On inspection of these curves it can be seen that they differ considerably from the normal. The most striking change is the general lowering of acid values obtained and the slowly climbing type of curve, especially in the earlier specimens. The volume of juice is also considerably lowered.

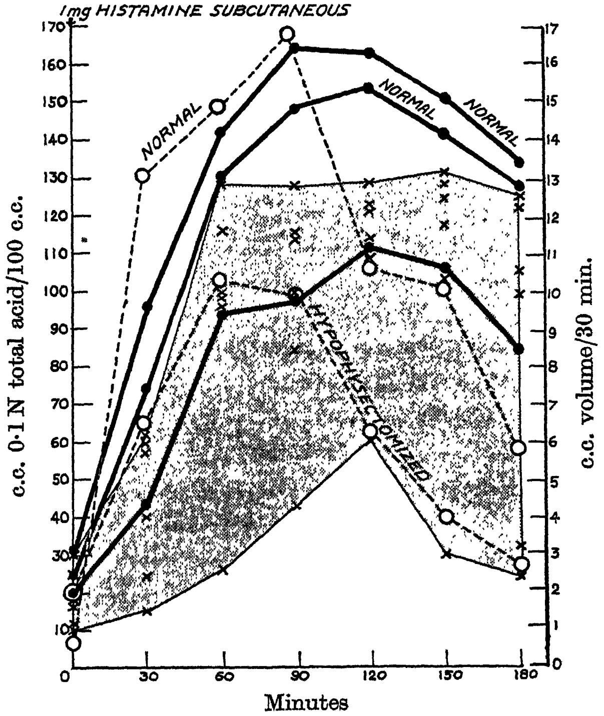

FIG. 2—Gastric secretion in hypophysectomized unanaesthetized cats. Histamine stimulus.

—•— c.c. 0·1 N total acid/100 c.c. ----o---- c.c. volume/30 min.

One operated cat at post-mortem was found to have a normal posterior and intermediate lobe of the pituitary, but only an insignificant fragment of anterior lobe. This cat had lost weight and showed atrophy of the testes and adrenal glands typical of anterior insufficiency. The response of the animal was, however, within normal limits.

On examination of all the results obtained on hypophysectomized animals using histamine as a stimulus, it was noted that, while many of the early 30 min. specimens had a low acidity, the volume of the juice secreted was very large. In normal cats a very definite relationship is found between the volume of juice secreted and its acidity. In fig. 3 this is seen by plotting the volume of every 30 min. specimen against its acidity. By the time the volume of juice has reached 3 c.c. the acidity of the juice has rapidly reached a high level where it is maintained as the volume further increases. A similar graph (fig. 4) for the hypophysectomized animals shows a striking alteration, and a regular curve is not obtained. The normal relationship between acid and volume has been completely altered. Many specimens of large volume have been obtained with relatively low acidity.

INSULIN STIMULUS

Seven tests have been performed on six hypophysectomized cats using insulin as a stimulus. The results are given in fig. 5. As the pituitary is known to exert an antagonistic action on the blood-sugar lowering effect of insulin, it would be expected that in these hypophysectomized animals, gastric secretion which is initiated by a fall in blood sugar would be more readily induced. That the blood sugar is more markedly affected is shown by the fact that whereas 2 units of insulin in a normal cat produce few symptoms, in these hypophysectomized animals convulsions occur and sugar administration is necessary to save the animal's life. The gastric effect, however, is lowered rather than increased. The volume of juice secreted is found to be negligible when compared to the control series. The acidity is probably within normal limits, the most marked change being that the 30 min. specimen contains higher acid values than that in the controls. The earliest at which this change has been observed is 4 days after hypophysectomy.

Two animals having an intact posterior lobe but only small portions of anterior lobe have been tested. One of these is the cat described above—HC 55—the other, HC 56, had even smaller remnants of anterior lobe. This was tested and killed 14 days post-operative, and the testes and adrenal glands were markedly atrophied. The response to insulin in these animals was quite normal.

SHAM FEEDING

The effect of hypophysectomy on the gastric stimulation produced by sham feeding is given in fig. 6. The normal response of the same animal is also shown. A similar test was carried out on cat HC 56—an animal described above with removal of the anterior lobe. On the completely hypophysectomized cat the normal volume of juice is not obtained from

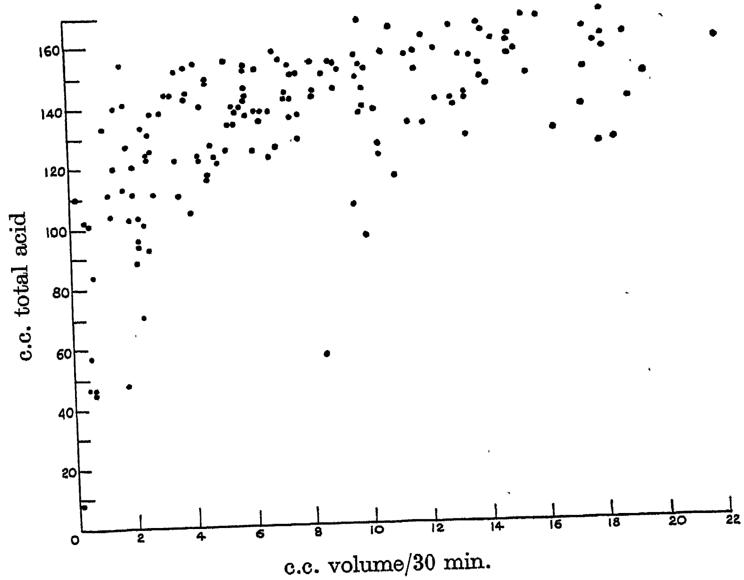

FIG. 3—Normal relationship of acid to volume of gastric juice. Histamine stimulus.

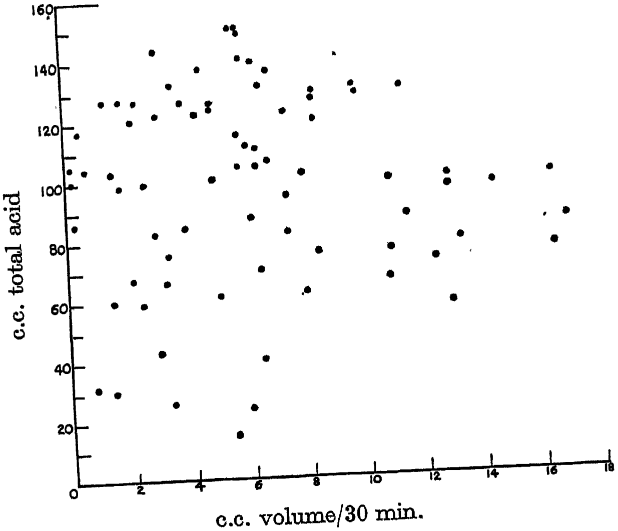

FIG. 4—Relationship of acid to volume of gastric juice following hypophysectomy. Histamine stimulus.

the stimulus, whereas in the cat with a posterior lobe the results were normal.

The experimental effects of removal of the pituitary on gastric secretion have been shown to consist of an alteration in the volume of juice secreted and resulting changes in acid curves. The most potent stimulus, histamine, produces generally lowered total values, but also an alteration in the relationship of acid to volume. Other weaker stimuli fail to evoke a secretion comparable with the normal. Anterior lobe removal alone does not produce these changes. These results show that the posterior lobe of the pituitary gland has a normal part in the regulation of gastric secretion. From the important role which blood flow has been shown to play in secretory activity, it would appear probable that the effects of hypophysectomy on secretion are due to an alteration in blood flow to the stomach.

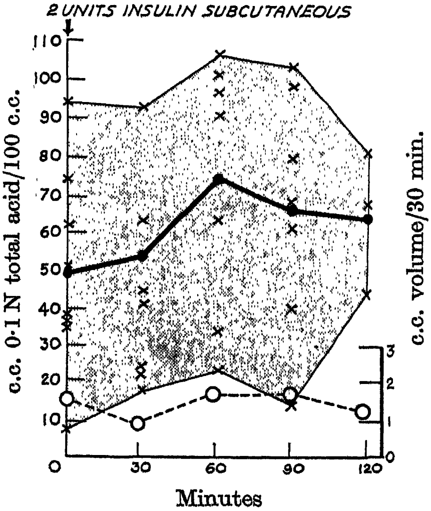

FIG. 5—Gastric secretion in hypophysectomized cats. Insulin stimulus.

—•— c.c. 0·1 N total acid/100 c.c.
----o---- c.c. volume/30 min.

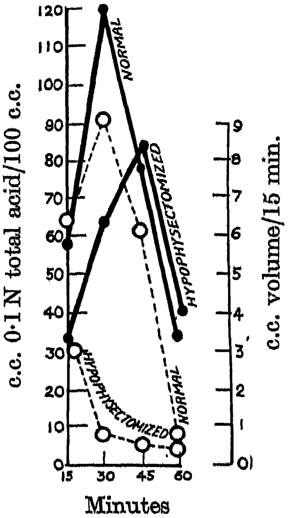

FIG. 6—Gastric secretion in hypophysectomized cat. Sham feeding stimulus.

—•— c.c. 0·1 N total acid/100 c.c.
----o---- c.c. volume/15 min.

BLOOD FLOW

A series of tests have been made measuring the blood flow and gastric secretion with hypophysectomized cats, the technique being the same as that described in the previous paper. The animals used had the pituitary gland removed at least 14 days previous to the experiments.

The results of these tests using histamine as a stimulus may be seen from Table I. When compared with the normal it is found that in general there is an alteration in the relationship of gastric secretion to blood flow. In some cases the blood flow may increase out of all proportion to the secretion produced. In others, the volume of the secretion may fall off while the blood flow is increasing. Occasionally normal secretion is seen, but the blood flow falls off throughout. Although no uniform curve is found after hypophysectomy, the relationship of secretion to blood flow is altered from that which occurs in the normal animal.

GENERAL DISCUSSION

The investigations described in this and the preceding three papers arose from the observation that the injection of an extract of the posterior lobe of the pituitary gland was capable of producing a severe lesion in the acid-bearing area of the stomach of the rabbit. In the earlier stages the experiments were designed solely with a view to solving the problem of the causation of this lesion, but it soon became apparent that the role of the posterior lobe of the pituitary body was even more complex than had originally been suspected, and that its secretion exerted a controlling influence on glandular activity throughout the body. The problem was attacked by the administration of an excess of the extract, and by a study of animals from which the gland had been removed.

The administration of the extract to normal intact animals leads to a series of definite and consistent changes in the nature of their glandular secretion. In the case of the stomach, there is first of all a partial inhibition of the gastric secretion resulting in a reduction in the amount of juice produced. The composition of this secretion does not vary very much, and the concentration of acid is usually unaffected. It is the total quantity or volume per unit of time which is reduced. If still more of the extract be administered, then complete inhibition occurs and it is impossible to detect the secretion of any fluid by the stomach. If the contents of the stomach in which secretion has been inhibited by this means be strongly acid, then a severe destruction of the gastric mucosa occurs with the production of the typical haemorrhagic lesion. This may heal perfectly, or it may heal irregularly and produce chronic lesions taking the form of an ulcer, and subacute or chronic perforation of the stomach may result. Experimental enquiry has established that two factors are necessary for the production of this gastric lesion. First, inhibition of secretion, and secondly, the presence of free acid in the stomach. It would appear reasonable to assume

TABLE I

Time min.	HC 64			HC 65			HC 67			HC 69			HC 80		
	Flow*	Volume	Total acid	Flow	Volume	Total acid	Flow	Volume	Total acid	Flow	Volume	Total acid	Flow	Volume	Total acid
0	18·0	1·5	40	—	—	—	—	—	—	22	—	79	28	1·0	43
15	10·6	1·6	49	15·0	—	—	9	0·3	37	22	0·35	74	21	0·45	46
0†	10·7	0·6	59	11·0	0·01	—	7	0·1	35	16	0·25	82	10	0·25	46
15	8·3	0·4	65	10·0	0·3	43	7	—	—	16	1·05	92	11	0·10	50
30	9·0	0·75	71	7·6	0·3	53	6	0·2	25	10	0·9	115	11	0·30	47
45	9·8	0·9	100	10·8	0·6	84	7	0·06	40	10	2·4	130	9	0·10	70
60	7·2	1·2	122	12·2	1·1	115	7	—	—	12	1·5	142	10	0·20	85
75	7·6	0·8	132	14·0	1·5	123	7	0·45	50	17	1·4	149	12	0·20	95
90	7·4	0·75	136	16·6	2·2	99	8	0·25	84	16	0·5	150	11	0·25	84
105	6·2	0·7	138	17·6	1·6	62	8	0·7	103	17	0·9	138	—	—	—
120	6·2	0·7	140	14·9	1·6	46	10	1·3	119	12	0·35	134	—	—	—
135	3·5	0·5	130	13·6	1·6	26	14	0·85	117	—	—	—	—	—	—
150	—	—	—	5·9	0·6	18	24	0·9	111	—	—	—	—	—	—
165	—	—	—	5·2	0·25	20	20	2·7	82	—	—	—	—	—	—

* Flow in c.c./min.

† 1 mg. histamine intramuscular.

that the stomach wall can only be protected from destruction by the continuous secretion of gastric juice, and that when this ceases the highly digestive fluid in the stomach is able to penetrate the mucosa and exert its destructive action. It is generally accepted that the constituents of gastric juice are secreted by different cells of the stomach mucosa; thus the hydrochloric acid would appear to be produced by the oxyntic cells, whilst the other constituents appear to come from the chief cells throughout the stomach. It will be remembered that the lesion only occurs in the area occupied by oxyntic cells; and since it has been demonstrated experimentally that only the volume of juice is inhibited, and not the acid secretion, it would appear reasonable to suggest that in the normal animal the fluid provided by the continuous secretion is responsible for the removal of the hydrochloric acid which is secreted by the oxyntic cells. The administration of an excess of pituitary extract stops this protective normal secretion of fluid, and a lesion results.

The mechanism of this inhibition has been fully investigated and it has been shown that a reduction in the blood flow to the stomach is the cause. Careful analysis of the phenomenon of gastric secretion has indicated that two factors are necessary: first, an adequate stimulus, and secondly, the ability of the vascular system to allow for a concomitant increase in the blood flow. It has been shown that one without the other is not sufficient to produce a secretion. In the normal animal there is a parallel relationship between the blood flow and secretion, the two factors rising and falling together.

Turning to the second method of investigation, namely a study of animals from which the gland has been removed, it would appear to the casual observer that there is little or no alteration in the stomach. Certain fine histological changes were observed and reported in the first paper, and certain gross lesions occasionally occur, but these will be referred to later. A careful study of gastric secretion in the hypophysectomized animal reveals at once a fundamental difference from the normal. In the first place the regular relationship between blood flow and secretion is abolished, and consequently the regular smooth relationship between acid and volume of gastric juice disappears. In the normal animal a rise in volume is associated with an increase in acidity, and vice versa; but it will be remembered, as pointed out earlier in this discussion, that the administration of an excess of posterior lobe extract cuts down the volume and leaves the acid unaltered. In the hypophysectomized animal this acid-volume relationship is abolished with the result that it is possible to find a low acid content with a high volume and the reverse. Experiments have shown that the explanation lies in the fact that in the hypophysectomized animal the vascular system of the stomach is not capable of allowing a regular increase in blood flow following the application of a stimulus, and what might be termed a disorderly secretion results.

In a few animals it is possible to see small haemorrhagic erosions in the acid-bearing area of the stomach. In a private communication (Parkes) it has been reported that in the ferret perforating ulcers have been encountered in a few hypophysectomized animals. Macroscopically, the lesion in the hypophysectomized animal presents a similar appearance to that produced by the administration of excess of the extract. No lesion, however, has ever been seen that approached in size and destructiveness that described in the injected animals. Whilst other possible causes are discussed in the first paper, a probable explanation for these lesions in the hypophysectomized animal may be that since the regular relationship between acid and volume is destroyed, a local condition might occur in which acid was secreted without an adequate amount of fluid to remove it.

In conclusion, these investigations indicate that the posterior lobe of the pituitary gland produces a substance which up to the present has not been dissociated chemically from the vasopressive principle, and which is necessary for maintaining the vascular system in such a state that it is capable of a smooth and regular activity, so that the blood flow to secreting glands such as the stomach may be co-ordinated with the secretion, following the application of an adequate stimulus.

These findings demonstrate a new relationship between the posterior lobe of the pituitary, the blood flow, and alimentary secretion; and they suggest an entirely new approach to research on diseases in which there is a derangement of the alimentary function.

CONCLUSIONS

Gastric secretion in hypophysectomized animals has been found to differ markedly from the normal.

The blood-flow response accompanying secretion is also altered from the normal.

These changes have been shown to be due to the absence of the posterior lobe.

The role of a substance secreted by the posterior lobe of the pituitary, essential for the normal regulation of secretion, is described.

During the course of this work we have to acknowledge with gratitude assistance from the following: The Council of the Middlesex Hospital Medical School for continuous support during this investigation. The Medical Research Council for a grant for expenses. Professor H. Selye and Dr. S. Baxter (McGill University) for the demonstration of operative technique. Mr. W. Lawson and Mr. G. M. Hills for the preparation of material. Messrs Boots Pure Drug Company and Messrs Schering, Ltd., for gifts of material, and Professor John Mellanby for a gift of secretin.

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A Study of the Medulla Oblongata of Cyprinodont Fishes with Special Reference to their Feeding Habits

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INTRODUCTION

It is well known that the hindbrains of fishes show considerable variation in size and structure. Drawing a correlation between the feeding habits and the structure of the hindbrains, Evans (1931–2) and Bhimachar (1935) have divided cyprinoid fishes into (1) *the mud feeders* which show enlarged vagal lobes; (2) *those which grope and grub for food* (always with barbels) showing prominent facial lobes; and (3) *fish which feed by sight at the surface of water* exhibiting very small vagal and facial lobes. To the last group belong the British roach, rudd, chub, dace and bleak, and the Indian rasbora, nauria, danio and chela. The purpose of this paper is to show that the cyprinodonts conform to this group of sight-feeding fishes. It was on account of the perfect surface-feeding habit of most of the cyprinodont fishes that Dr. Evans suggested to me that it would be worth while examining their medullae oblongatae. In a personal communication Professor C. J. Herrick states: "The correlation of habits of fishes with their anatomical structure is an easily accessible problem, and there is no point at which it can be attacked that will not yield new and interesting results. The field has scarcely been explored at all." Furthermore, an attempt is made to assign a definite position to the central acoustic area or lobe as a regional subdivision of the medulla oblongata.

MATERIAL

There are very few cyprinodont fishes in India. The forms that have been worked out in this paper are: *Gambusia affinis, Lebistes reticulatus, Haplochilus melanostigma* and *Panchax parvus*. *Haplochilus* and *Panchax* are Indian types; the other two are imported forms. They are all small, carnivorous and surface-feeding forms. The ground-feeding cyprinodonts which are confined to South America are not described here. The brains of the above forms have been fixed in Bouin's fluid and the sections have been stained in iron haematoxylin. The brains of a few specimens were fixed in 5 % potassium bichromate solution and the sections stained by the Weigert-Pal method and counterstained in paracarmine (modification of the method of Kappers). To illustrate the central acoustic lobe clearly the figures have been drawn from sections stained in iron haematoxylin. The Weigert material has been useful in studying the fibre tracts and their distribution clearly.

FEEDING HABITS

All the fish under consideration are surface forms and they feed mostly by sight on insects, insect larvae, and small Crustacea at the surface of the water. It is interesting to observe how, as soon as a cyprinodont sees the prey, it suddenly leaps towards it and devours it. Correlated with this habit the eyes are large, and the mouth is directed upwards, with flat jaws. Barbels form important gustatory appendages in those fish that grope and grub for food, but are absent in the sight-feeding cyprinodonts.

The top-minnow (*Gambusia affinis*) is a perfect surface feeder. It is extensively used by the Department of Health in Mysore and elsewhere for the control of malaria. It is a hardy fish and has been acclimatized to the local waters very thoroughly. It is said that one minnow destroys

165 large mosquito larvae in a single day. The food of this fish consists largely of insects and insect larvae; but sometimes it devours its own kind when there is not sufficient food available.

"Millions" (*Lebistes reticulatus*) is the only fresh-water fish in the island of Barbados. This is a smaller fish than the top-minnow. They have proved very efficient in destroying mosquito larvae. They readily attack small insects in water or resting on the surface of the water.

Haplochilus—Writing about the habits and habitat of this fish Sundararaj (1916) states that "It inhabits the shallows of ponds and rivers where it affects the surface and swims very close to it, frequently with its mouth touching it. When swimming in their natural surroundings, the surface of the head is kept almost on a level with the plane of water. The flat head and transverse mouth are adaptations to surface feeding and the species is well known as an effective mosquito destroyer. The habit of inhaling the oxygenated surface water enables it to withstand to a considerable extent foul water full of organic impurities detrimental to most fish: in the city it frequently occurs in gutters and sewage farms." *Panchax* has similar habits to *Haplochilus*, but it dislikes foul water and prefers stationary and sheltered waters of tanks and rivers overgrown with vegetation. The food of these fish consists mainly of insects which can readily be made out when their stomach contents are spread on a slide.

STRUCTURE OF THE BRAIN

A naked eye examination of the brain of the top-minnow shows the presence of prominent optic lobes, a well-developed cerebellum and a narrow tapering medulla oblongata. The brain in *Haplochilus* and *Panchax* is considerably compressed dorso-ventrally on account of the flattening of the head, so much so that its transverse diameter in a cross-section is double its height, in the region of the central acoustic lobe. As a type form the medulla of the top-minnow will be described in detail. The sections have been described from behind forwards.

Fig. 1 represents the section of the medulla of *Gambusia* passing through the vagal lobes at the entrance of the vagus nerve. The size of the vagal lobes is small when compared with those in other bony fishes in general. The upper visceral (visceral sensory column) and the lower motor (nucleus ambiguus) centres can be distinguished. The somatic sensory column, so prominently developed in the bottom-feeding forms (loach and carp), is not distinct. On the left side can be observed the gustatory fibres passing from the vagal lobe to the secondary gustatory tract. The section in fig. 2 passes through the rostral end of the vagal lobe and here can be seen the small glossopharyngeal lobe and the IX nerve also. The somatic sensory of the V lobe passes as far back as this region and it is highly developed in this fish. A number of well-myelinated fibres can be observed, particularly in the Weigert preparations, passing from the V lobe on either side and arborizing into the substantia reticularis. Substantia reticularis is the chief medium of communication between the sensory and the motor centres for simple reflex paths. The spinal V tracts which are seen in figs. 1 and 2 contain fibres coming from the general cutaneous system and represent the somatic afferent of the trunk (Herrick 1905).

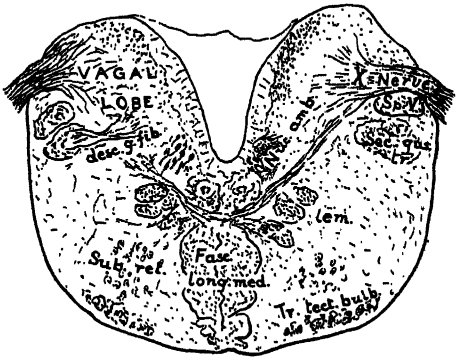

FIG. 1—*Gambusia.*

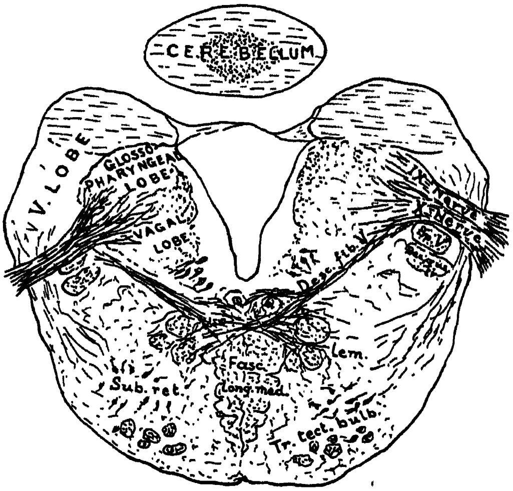

FIG. 2—*Gambusia.*

The facial lobes are small and cannot be seen from outside. Careful microscopic examination of the sections reveals two insignificant lobes embraced by the huge somatic sensory or V lobes (fig. 3). They are separate and do not coalesce with each other as in the cyprinoids. In size and position they are more or less similar to the small facial lobes of the whiting (Evans 1935). The facial nerve which enters the medulla transversely in the region of the central acoustic area (fig. 4) travels backwards medio-ventrally and then breaks up and distributes into the lobe. In the region of the facial lobe there is an arch-like thick bundle of deeply stained fibres connecting the two somatic sensory areas. This is the commissura infima. This does not receive any fibres from the visceral sensory centres of the vagal lobes. Except for this connexion the two somatic sensory areas are completely separate from each other. Lemniscus, which is the chief sensory secondary tract, can be seen in all figures on either side of the median longitudinal fasciculus. As it passes rostrally it receives thick bundles of fibres from the V lobes and the acoustico-lateral tubercles. The remarkable feature about the section in fig. 4 is the presence of a prominent transverse small-celled area situated below the cerebellum. It corresponds to the central acoustic area described by Evans in 1932. This area is regarded as the terminal centre in the brain for the sense of hearing. This area is quite

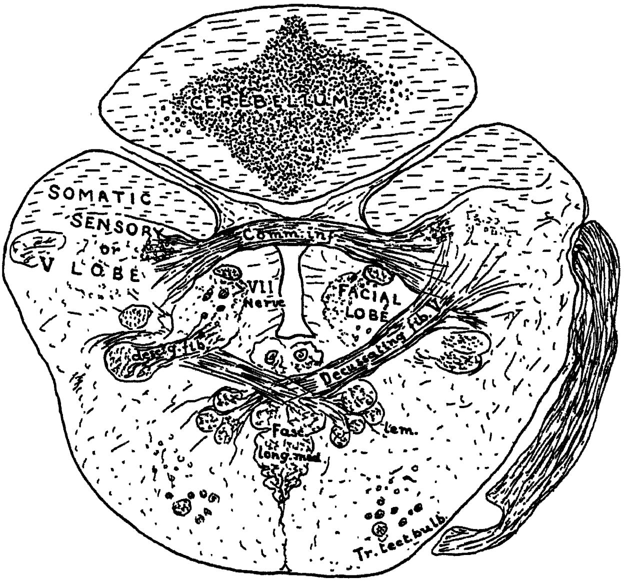

FIG. 3—*Gambusia.*

separate from the cerebellum. As we pass rostralwards through a few sections we observe that the small rounded cells of the central acoustic area become grouped into two sets separated by a central cell-less area, and finally nerve fibres from these pass into the respective acoustico-lateral tubercles. Lying below the central acoustic area and into the ventricle is a single layered ring-like structure (fig. 4, *V.S.*). This is the cross-section of a sac which arises as an infolding of the epithelium which forms the roof of the ventricle just in the region of the commissura infima and projects forwards into the ventricle as a blind sac. Its wall is composed of a single-layered epithelium of large cells. The exact nature of this sac could not be determined.

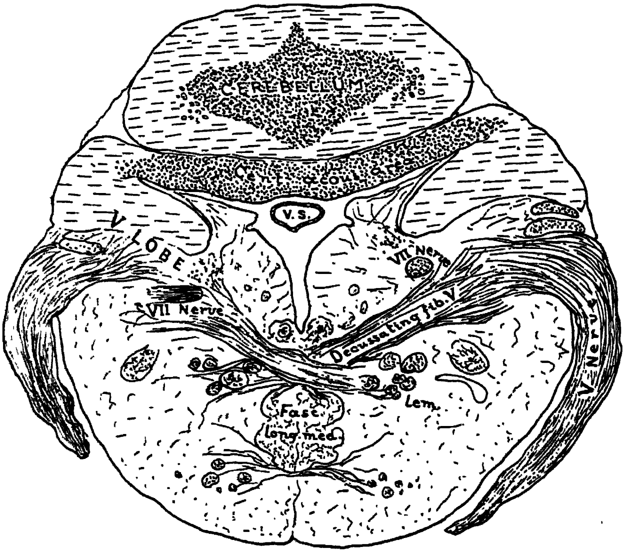

FIG. 4—*Gambusia.*

In general organization the hind brain of *Lebistes*, *Haplochilus* and *Panchax* resembles closely the medulla of *Gambusia* and it is superfluous to describe them in detail with figures. It is necessary to give only the figures (figs. 5 and 6) of the cross-sections of the brains of *Haplochilus* and *Panchax* in the region of the central acoustic lobe.

It must be mentioned here that as a result of the sight-feeding habit of the cyprinodont fish at the surface of water the medulla oblongata is narrow, without prominent facial or vagal lobes, and that there has developed a conspicuous central acoustic area.

DISCUSSION

This investigation offers two points for discussion. The surface-feeding habit of the fish has led firstly to the poor development of the facial and the vagal lobes and secondly has stimulated the growth of an auditory centre in the brain—the central acoustic area or lobe.

It is easy to explain the first point if we realize that these fish feed primarily by sight and consequently the gustatory organs (taste buds) are poorly developed, with the result that the correlation centres of the gustatory organs, the VII and the X lobes, are very small. In the cyprinoid and siluroid fishes, which possess taste buds all over the body and especially on the head and barbels, there is an enlargement of the VII, IX and X sensory nuclei. Although the facial and the vagal lobes are small in the cyprinodonts, the V or the somatic sensory lobe is very prominent. This is probably due to the more active life of the fish. Being sight feeders they

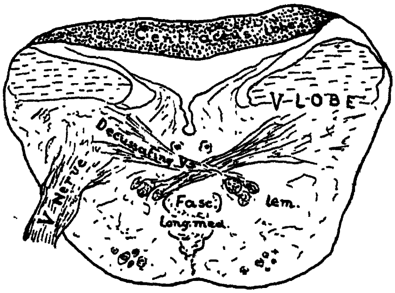

FIG. 5—*Haplochilus*.

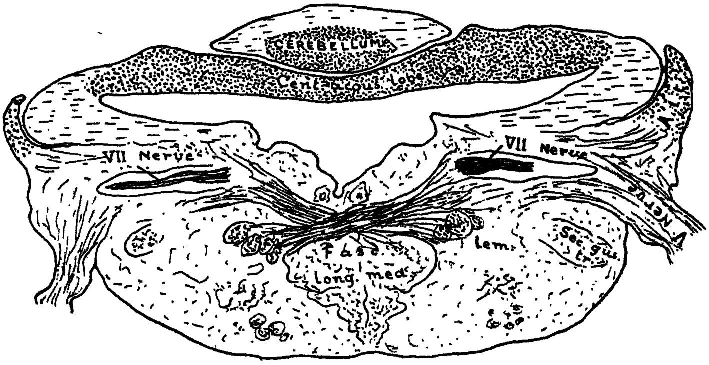

FIG. 6—*Panchax*.

have to be more active and alert than the bottom feeders. Evans (1935) concludes that the presence of the enlarged somatic sensory lobe of the whiting is due to its predaceous habits.

The second point, namely the presence of the central acoustic area or lobe and particularly its auditory function, has to be discussed with caution. It has been clearly shown by Evans (1932) and Bhimachar (1935) that the central acoustic area or lobe is highly developed in surface-feeding fishes. The explanation that has been offered for the development of an acoustic area in such forms is that as a result of constant exposure to the external sounds at the surface of water they have acquired a keen sense of hearing. While the ears hear the external sounds, tones of low frequency are perceived by the lateral line sense organs.

Whether the fishes hear at all with their auditory organs had been doubted for a long time. But the experiments of Parker (1918) and Von Frisch (1931) have shown clearly that the fishes do hear sound waves with their ears. Evans (1932) quotes an excellent instance of hearing of a fish in herring. The sense organ responsible for hearing in fishes is the saccule and lagena complex, from a part of which the cochlea of the higher vertebrates has evolved. In the atherinid fish *Menidia*, a distinct branch of the VIII nerve supplying the lagena has been demonstrated by Herrick (1899). In his article on "Can Fish Hear?", Bull (1930) has described the experiments of H. Stetter on the sense of hearing of fishes. Stetter has been able to train fishes to come to a definite part of the aquarium for receiving food, on hearing certain sounds. After a number of lessons the fish would come to that spot on hearing the sound even if the food was not given and exhibit what Stetter calls "food reactions". In the case of a minnow he had removed the eyes to avoid error by sight and the fish showed remarkable response on hearing the sound. He has pointed out clearly that the capacity to hear varies with different fishes.

The acoustico-lateral area is a highly complex structure in the brain of the fish. Not only do the fibres of the VIII nerve representing two distinct functions (auditory and vestibular) but also all the fibres of the lateral line nerves conveying a third type of impression terminate in this nucleus. To explain this point we cannot do better than quote Herrick (1924), "The acoustico-lateral area of fishes receives all nerve fibres from the internal ear and from several kinds of lateral line organs, and the central terminations of these different sets of fibres are so intertwined within this area that it has hitherto not been possible to separate completely the reflex centres of many diverse functions represented in this complex system of peripheral sense organs. This is, however, an incomplete separate localization within this area of several specific functions, but the reflexes served by all of the organs of this complex are in close physiological association. There are three groups: (i) equilibratory and postural, served chiefly by the semicircular canals of the ear; (ii) auditory, served by the end organs of the saccule; (iii) reactions following excitations of the lateral line organs by slow water vibrations." Any advance in our knowledge of this important part of the brain should be to mark out broadly the three functional reflex centres.

We owe our knowledge of the central acoustic area or lobe in fishes to Evans who was the first to attribute an auditory function to this nucleus in the brain. In all fishes which possess central acoustic areas or lobes there are distinct bundles of nerve fibres passing from inside the lobe, on either side, to the acoustico-lateral tubercles. Furthermore, Evans (1932) has shown clearly by a process of elimination that this part of the brain has no relationship either with the lateral line sense organ or with the swim bladder. The evidence in favour of the auditory function of this area is almost conclusive. It is desirable to try to trace the auditory part of the VIII nerve entering and distributing itself into the central acoustic area. If this could be achieved, which is no doubt a difficult task, a definite area for the sense of hearing will have been established in the piscine brain. At the present state of our knowledge of this part of the brain it is rather difficult to say whether it has not also some relationship with the vestibular part of the internal ear in addition to the sense of hearing.

I take this opportunity of expressing my gratitude to Dr. H. Muir Evans and Professor C. R. Narayan Rao for their valuable suggestions and help, and to Dr. B. Sundararaj and Dr. B. Thirumalachar for kindly sending me specimens for this work.

SUMMARY

The cyprinodont fishes, in general, feed largely by sight on insects, insect larvae and other organisms at the surface of water. As a consequence of this habit the eyes are large, and the mouth is directed upwards with flat jaws. The gustatory system is of secondary importance and is poorly developed, with the result that the vagal and facial lobes are small. There is an enlarged somatic sensory or V lobe. Since these fish are constantly exposed to external sounds at the surface of water, their auditory centre in the brain—central acoustic area or lobe—is well developed.

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ABBREVIATIONS

A. lt.	Acustico-lateralis area.
Cent. acous. area	Central acoustic area.
Cent. acous. lobe	Central acoustic lobe.
Comm. inf.	Commissura infima.
Desc. fib. V	Decussating fibre V.
Desc. g. fib.	Descending gustatory fibres.
Fasc. long. med.	Fasciculus longitudinalis medialis.
Lem.	Lemniscus.
Nuc. amb.	Nucleus ambiguus.
Sec. gus. tr.	Secondary gustatory tract.
Sp. V	Spinal V tract.
Sub. ret.	Substantia reticularis.
Tr. tect. bulb.	Tractus tectobulbaris.
V.S.	Ventricular sac.

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The Change of Visual Sensitivity with Time

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1—Introduction

On changing instantaneously the external light stimulus applied to the eye, the latter does not also change instantaneously its state of adaptation to that corresponding to the new stimulus. No general definition of the state of adaptation of the eye has been given, or, perhaps, can be given. One can, however, imagine quite clearly that, when the eye is viewing some given external distribution of brightness, the concentration of photochemical substances in the various parts of the retina, the nervous messages sent from retina to brain and the consequent reactions in the latter will all be tending towards certain equilibrium values. When the external brightness distribution is changed, these equilibrium values will also change in the direction of a new set of equilibria. Unfortunately, it is difficult to measure any of these things directly and it is necessary to compromise. Amongst quantities more readily measurable the liminal or threshold responses of the eye occupy an important place. These quantities must be closely related to the actual physical state of the visual system and they are of considerable practical significance in themselves. The problem of the change of visual state with time has therefore been approached by measuring the change of visual sensitivity with time.

The general experimental problem is to expose the eye to a certain primary or conditioning stimulus for a given period, then suddenly to change to a different stimulus, called for convenience the secondary stimulus, and to make at intervals thereafter a series of threshold measurements, the secondary stimulus remaining steady. This general specification of the problem may be subdivided as follows:

1—Primary and secondary stimuli the same in pattern.
  (*a*) Primary stimulus greater than secondary.
  (*b*) Primary stimulus less than secondary.

2—Primary and secondary stimuli different in pattern.
This section may also be subdivided according to whether the secondary stimulation corresponds to (*a*) a higher, (*b*) a lower, or (*c*) the same equilibrium value of the visual test on the test area of the retina which is under investigation.

The above classification is to be further multiplied by the number of different visual tests which can be used and by the number of different retinal test areas. The ramifications of all the possible different colour combinations of stimuli and tests must also be considered. This leads to an almost infinite number of possible experiments, but the number which has theoretical and practical interest is limited, though still very large.

Cognisance must also be taken, especially in relation to practical applications, of the fact that the intrinsic sensitivity of the retinal system is not the only thing which changes with the external light stimulus. The pupil of the eye varies in size and, though the variation has an effect small in comparison with the retinal sensitivity variation, it is a factor to be taken into account in any practical application of the result. It has been considered the more satisfactory to investigate retinal sensitivity and pupil size separately, combining the results when desired, and the question of pupil size variation and related matters are considered elsewhere. All measurements of retinal sensitivity changes have therefore been made using a fixed artificial pupil smaller than the smallest natural pupil to be encountered in the experiments.

2—GENERAL EXPERIMENTAL CONDITIONS

The work to be described in what follows falls under section 1 (*a*) of the above classification. The visual test used is the least difference of brightness perceptible to the eye. In the absence of a general background illumination this becomes the least brightness perceptible. Monocular vision is used, the unemployed eye being in darkness. The colour of light in all fields is white, of colour temperature approximately 2700° A. The disposition of the field of view is shown in fig. 1. The central portion, of diameter subtending at the eye an angle of about 45°, can be uniformly illuminated to any brightness level up to 130 c./sq. ft. The rest of the field is more or less in darkness, being painted black and receiving only scattered light from the background. The test area is a small circular patch of light projected on to the centre of the background *A* and exposed intermittently, the flash period varying from 1 sec. to $\frac{1}{20}$ sec. As an alternative to the uniform background of 45° diameter the image of a small source of light may be made to appear at D, the angle between A and D being variable within limits. The illumination at the eye due to this glare source at D may have any value up to 60 ft.-c. When the test area is to fall upon the fovea, location points B, B are projected on the background so that A is midway between them. The subject then has to imagine the point midway between B and B and fixate on it. For testing extrafoveal retinal areas a single fixation point C is used at the required angle from A, the subject fixating upon it directly.

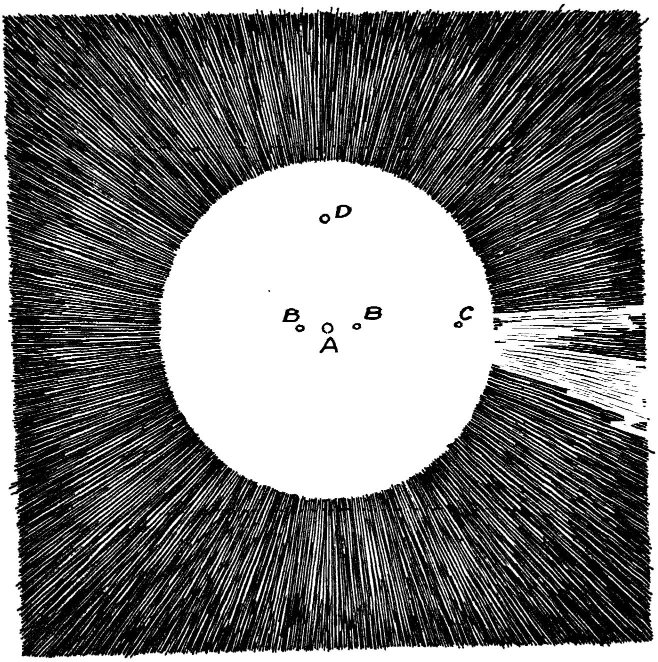

FIG. 1—Disposition of subject's field of view.

Mechanism is provided whereby the uniform background or the glare source may be suddenly cut off or reduced in intensity and threshold determinations made at a series of known intervals afterwards. The exact procedure to be adopted depends upon the length of the interval. Three methods have been used: (*a*) for intervals of 1 min. upwards, (*b*) for intervals of 1–100 sec., (*c*) for intervals less than 2 sec.

Method (*a*)—The subject looks at the primary conditioning stimulus for a period sufficient to attain approximate equilibrium (5 min. is generally sufficient unless the conditioning stimulus is very low and the eye has been previously exposed to a high level), fixating in the appropriate manner.

The change to the secondary stimulus is then made at a known instant and threshold determinations made periodically thereafter at known times. These determinations are made by the "multiple decision method" as used for static conditions. The test-spot, initially just definitely visible, is exposed every few seconds and reduced in intensity after every flash. The subject records by electrical marker whether each flash was visible or invisible. In due course the test-spot is no longer seen; it is then made to increase in intensity after each flash and the record of "visible" or "invisible" is continued. The time of change over from decreasing to increasing intensity is noted and taken as the time of the threshold determination. The double record shows the test-spot as visible at one end, invisible at the other, with a stretch of uncertainty in the middle. The point is chosen for which there is the same number of "visibles" on the invisible side as there are "invisibles" on the visible side, or, as it might be termed, the centre of gravity of the region of uncertainty. Preliminary calibration enables the threshold intensity corresponding to this point to be determined. Threshold measurements are repeated in similar fashion at suitable intervals up to the limit of time desired. This method breaks down for intervals less than about a minute.

Method (*b*)—The subject determines the time required for a fixed threshold intensity to become visible. As in (*a*) he looks first at the primary conditioning stimulus for a suitable period, after which it is changed to the secondary stimulus. Thereafter the test-spot of constant intensity is exposed at known equal intervals until it is definitely visible. A record is made at each exposure of "visible" or "invisible" as in (*a*) and the record is treated in the same way. It is, of course, only a one way record, the test-spot appearing more and more distinctly as time proceeds, but the estimate of threshold thus obtained is the only one possible. Also, as mentioned in the paper by Stiles and Crawford (1934, pp. 66–8), the threshold value obtained by the multiple decision method is, on the average, independent of whether the test-spot is increasing or decreasing in intensity. The whole procedure has to be repeated for each of a series of different test-spot intensities and is applicable to intervals of 1–100 sec. These are extreme values, for the test-spot visibility is very difficult to follow if it is exposed more frequently than once a second, and the eye becomes fatigued and uncertain in its response if the interval is too long.

Method (*c*)—For the shortest intervals a method has been developed using the multiple decision method in conjunction with a cyclic repetition of events. The primary conditioning stimulus and the secondary stimulus are exposed alternately, each for a given period, the cycle of events being maintained automatically. The test-spot can be exposed at any time during a cycle, at an interval after the change from primary to secondary stimulus which is constant for a given setting of the apparatus. After each exposure of the test-spot, the latter is automatically reduced (or increased) in intensity by a certain ratio. Records of "visible" or "invisible" are made and treated as in method (*a*). This cyclic method has one obvious defect which limits its application. The individual cycles cannot be made too long or the whole experiment would become impracticably tedious. Hence the method can only be used for comparatively short times. If the secondary stimulus occupies more than a tenth of the cycle, the state of the eye at the end of each primary stimulus begins to diverge appreciably from the equilibrium state and the conditions of the experiment are not fulfilled. A cyclic period of about ½ min. has been found to be the longest which can be conveniently used; this allows a maximum interval of about 2 sec. between change from primary to secondary stimulus and exposure of test-spot.

3—Apparatus

The general features of the apparatus are shown in plan in fig. 2. Part has been previously described (Stiles and Crawford 1933; 1934, p. 55), but for convenience the whole description is briefly given here. The nucleus of the apparatus is a cubical box *A* of about 2 ft. side. The subject looks through an aperture *B* in one side of the box and observes the opposite side, which forms the general background screen. This side is of metal, coated with magnesium oxide; all else is painted dull black. Aperture *B* has a diameter of about 3 mm. and forms an artificial pupil smaller than the natural pupil of the subject's eye. The background is illuminated by two lamps *C*, *C′*, images of which are formed by lenses *D*, *D′* in apertures *E*, *E′*. Filters may be placed at *E*, *E′* for regulation of background brightness. At *E*, in addition, there is an electrically worked shutter to open or close the aperture.

A small hole *H* in the background admits the image of a 500 c.p. pointolite lamp *F* focused within it by the lens *G*. This provides the glare source *D*, fig. 1. It is, actually, above the median plane of the apparatus. In the glare source beam is an electrically worked shutter similar to that at *E* and a holder for neutral filters for intensity regulation.

The beam of light producing the test-spot originates at the 100 c.p. pointolite lamp *K*. An enlarged image of the main electrode is focused in the plane *M*, where various apertures may be fixed. The beam then passes through neutral filters and a neutral wedge *N*, is reflected at mirror *O*, converged by lens *P*, reflected again at prism *Q* and finally forms on the background screen an image of the aperture at *M*. The test-spot beam from *K* to *Q* is slightly above the level of aperture *B* so as to clear the subject's field of vision, being deflected downwards at *Q* so as to fall on the centre of the background screen. Another electrically controlled shutter is inserted in the test-spot beam near *M*. This and the timing apparatus are described in detail later.

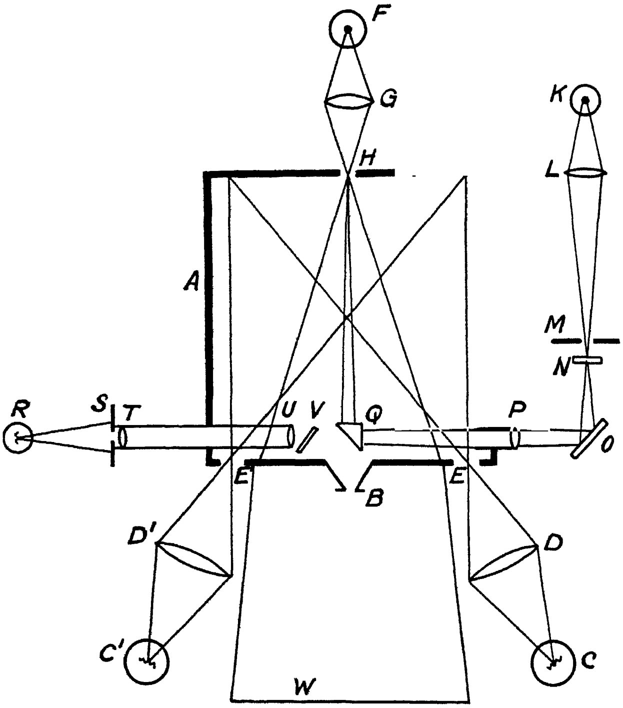

FIG. 2—General plan of apparatus.

The neutral wedge *N* is attached to a carriage moving vertically in guides. An electrically operated pen marker with two pens is also fixed to the carriage and moves over a paper tape on which the pens, controlled by the subject of the experiment, record whether the test-spot is visible or invisible at any particular point of the wedge. A fixed scale beside the tape is calibrated in wedge densities. After each test-spot exposure the wedge is moved automatically through a predetermined distance; a hand adjustment is also provided.

The optical parts for the projection of fixation points on the background are a 100 W projection lamp *R*, an aperture *S*, a condenser lens *T* forming an image of lamp *R* at a projector lens *U*, and a mirror *V* which reflects the beam on to the background screen. The projector lens forms an image of the aperture *S* on the background and the mirror is used to adjust its position as desired. The intensity of the fixation points is regulated by varying with a resistance the voltage applied to the lamp *R*.

All measurements of background brightness, test-spot brightness and illumination at the eye due to the glare source are made from the eye position by the subject. Brightness measurements are made by sighting an illuminometer directly upon the appropriate part of the background screen. Eye-illumination measurements are made by measuring with the illuminometer the brightness of a transmitting test plate (thin flashed opal glass) mounted vertically at the eye position. The test plate is calibrated by measuring its brightness when at a known distance from a lamp of known candle-power. Brightness is expressed throughout in c./ft.^{2}, illumination in ft.-c.

When the subject is viewing a field of very low or zero intensity great care is necessary in the elimination of stray light. The box *A* is light tight except for the holes for observation and for introduction of the various beams of light. Holes *E*, *E'* and *H* are closed by shutters when not in use. All lamps are surrounded by screens of black cloth or black painted tin and the room containing the apparatus is painted black. In addition, the subject sits in a little enclosure of black curtains hanging nearly to the floor. Warm or cold air for ventilation is blown in through the roof of the enclosure.

The shutter in the test-spot beam and the associated timing mechanism are shown diagrammatically in fig. 3. The shutter is a sector disk *A* running at constant speed checked stroboscopically by a segmented disk *B* and neon lamp *C*. A single transmission of the test-spot beam by the sector opening is picked out by an auxiliary electromagnetic shutter *D* energized through contacts on a drum *E* driven by reduction gearing from the primary sector spindle. The gear ratio must be 1 : some integral number; 1 : 7 is actually used, this and the rest of the gearing being meccano parts. The drum is a solid ebonite cylinder, 2 in. in diameter, with thin copper or brass segments screwed on the surface, contact being made by phosphor bronze or copper strips. The left-hand contact opens the shutter *D* just after the sector opening has passed across the beam, the right-hand contact closes it just after the sector opening has passed across the beam for the second time. This two-way working of the shutter was adopted in order to obtain a positive movement in both opening and closing. The arrangement as described so far would give an exposure of the test-spot at every seventh revolution of the sector disk. At the normal sector disk speed of 5 rev./sec. this results in an exposure every 1·4 sec. In order to obtain exposures at longer intervals two further contact drums *F*, *G* are used geared together as shown. The gear ratios are 1 : 3 between the second and third spindles, 1 : 7 between the third and fourth. Two switches *H*, *J* are arranged to short across the contacts on *F* and *G*. Synchronism of the various drums is arranged so that with *J* closed and *H* open every third revolution, with *H* closed and *J* open every twenty-first revolution of drum *E* is effective in working the test-spot shutter. This gives intervals between test-spot exposures of 4·2 sec. and 29·4 sec. respectively.

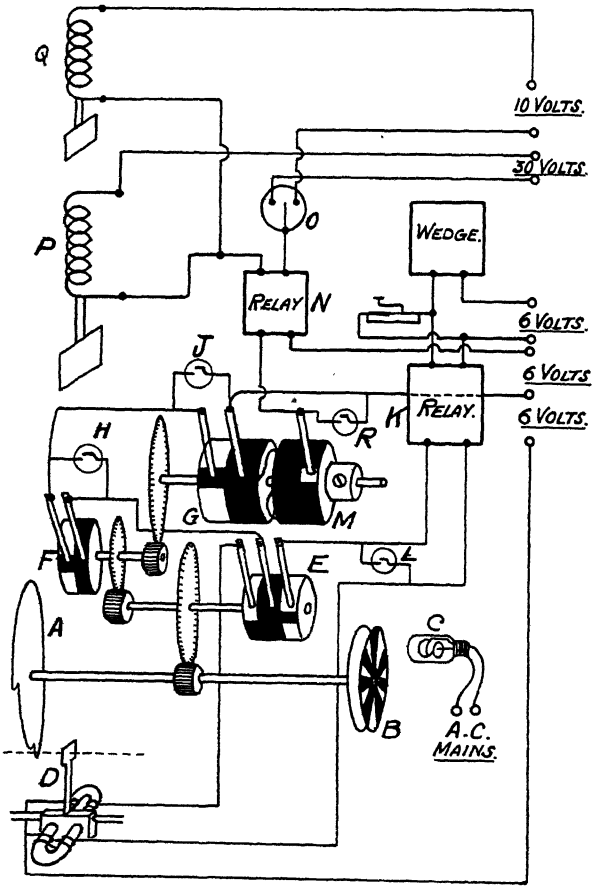

FIG. 3—Timing apparatus.

The movement of the wedge in the test-spot beam is controlled by a relay *K* in series with the "off" coil of shutter *D*. When energized immediately after an exposure of the test-spot the relay closes an electric circuit for a short period and raises or lowers the wedge through a certain distance by an electromagnetic release.

The synchronizing of the background and glare source with the test-spot shutter depends upon a contact drum *M* mounted upon the same spindle as drum *G*, but capable of being rotated and fixed in any position relative to *G*. The contact on *M* is in series with a relay *N* which, through a two-way switch *O*, controls either the background shutter *P* or the glare source shutter *Q*. The exact interval between test-spot exposure and make or break of the contact on *M* is estimated by counting revolutions and fractions of a revolution of the sector disk spindle between the two events. This eliminates effects due to backlash in the gearing but does not allow for lag in the shutters, which finally limits the shortest interval obtainable with this apparatus.

4—RESULTS

The results to be described below are of a somewhat preliminary nature, an exploration of various parts of the possible field of investigation to ascertain the general nature of the ground, and they do not in all cases approach finality. Not that finality can actually be reached in any measurement, but the errors are very large in these threshold measurements, the subject generally showing irregular changes in response from time to time, and large numbers of individual measurements are required to disentangle effects of real significance from these fortuitous variations.

4·1—*Variation of Brightness Threshold after Change from a Uniform Background Brightness to Zero*

A few measurements under this heading have already been published (Stiles and Crawford 1932); comparison with the present results will be made later in the section on the application of the equivalent background brightness hypothesis. The present results refer to two subjects, the test-spot falling firstly upon the fovea, secondly upon a small area 5° from the fovea, thirdly upon a small area 14° from the fovea. The test-spot was round, of diameter 0·46°, exposed for 0·05 sec. The same test-spot is used also in §§ 4·2 and 4·3. In the case of one subject four different initial background brightnesses were used, for the other subject only the highest. The results are shown in figs. 4–6, the continuous lines being those for subject

B. H. C., the dotted lines those for subject H. R. S. In each case (and also in the majority of subsequent curves) the logarithm of the brightness threshold is plotted against the logarithm of the time in seconds after the change to zero background brightness. The specification of retinal positions refer to the external field of view. Against each curve is shown the value of the background brightness to which the subject's eye was first adapted. Each curve is the smoothed mean curve through points from two or more

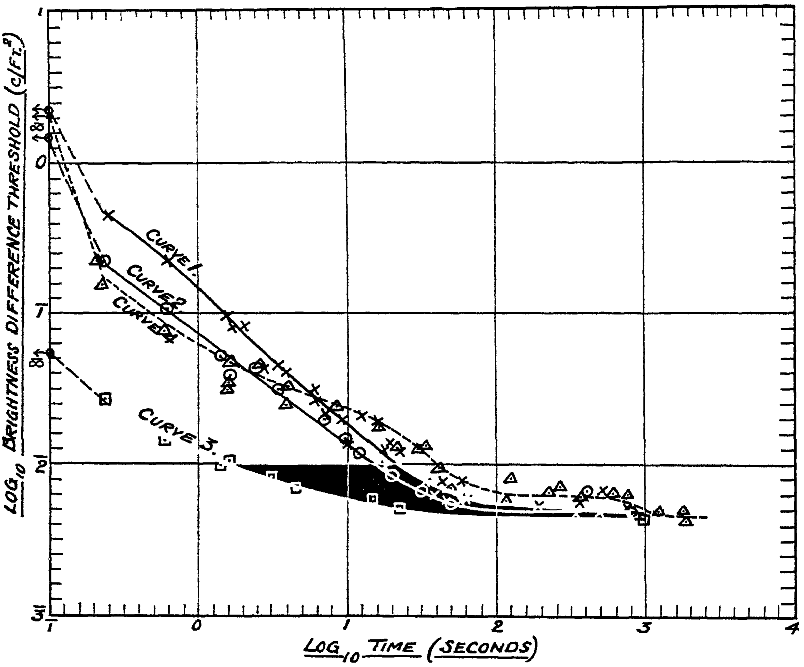

FIG. 4—Variation of brightness threshold with time after changing from various background brightnesses (B_0) to zero for a test-spot falling on the fovea. Subject B. H. C., curve 1, $B_0 = 132$ c./ft.²; curve 2, $B_0 = 78$ c./ft.²; curve 3, $B_0 = 1{\cdot}55$ c./ft.². Subject H. R. S., curve 4, $B_0 = 78$ c./ft.².

complete runs. The initial point for each curve at zero time is the threshold value for the steadily exposed background.

The logarithmic threshold and time scales give the most generally convenient and compact presentation of the results except for the shortest times, the defect being that zero time is at minus infinity. What happens at very short intervals after cutting off the background may be better illustrated by plotting the results on a linear time scale. When this is done for the data of figs. 4–6 it is seen that there is no indication of any discontinuous change of threshold on cutting off the background. The exploration of this region is very far from complete, however, as the experimental points start only at about $\frac{1}{4}$ sec. after cutting off the background.

It is interesting to compare the curves for subjects B. H. C. and H. R. S. which relate to the same primary conditioning stimulus (79 c./ft.^{2}). The

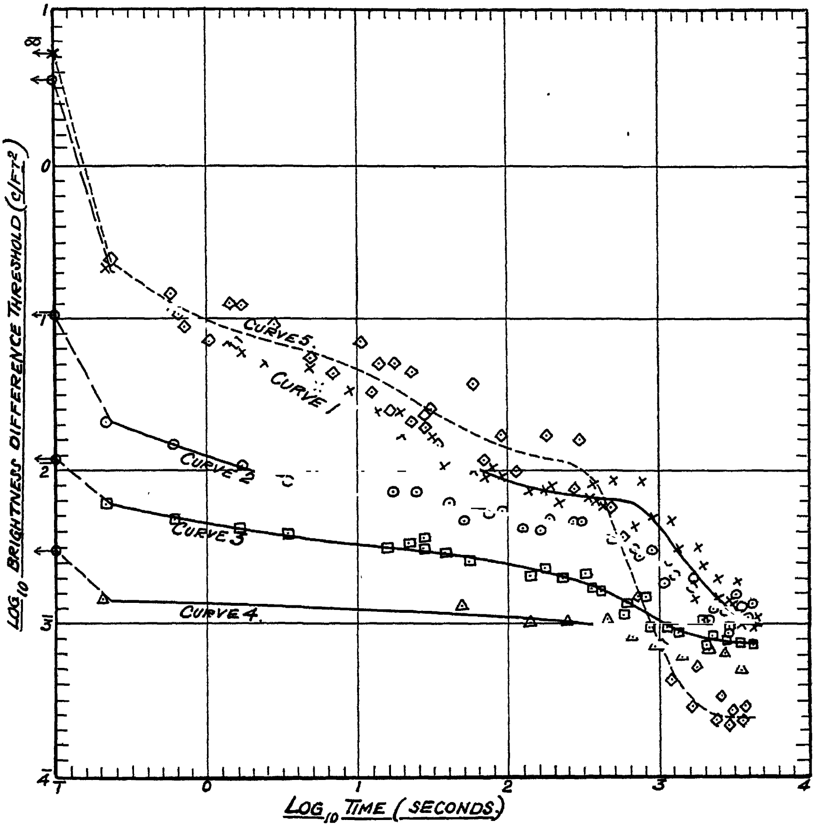

FIG. 5—Variation of brightness threshold with time after changing from various background brightnesses (B_0) to zero for a test-spot falling 5° from the fovea. Subject B. H. C., curve 1, $B_0 = 79$ c./ft.^{2}; curve 2, $B_0 = 1{\cdot}48$ c./ft.^{2}; curve 3, $B_0 = 0{\cdot}044$ c./ft.^{2}; curve 4, $B_0 = 0{\cdot}000174$ c./ft.^{2}. Subject H. R. S., curve 5, $B_0 = 79$ c./ft.^{2}.

curves for a foveal and for a 14° parafoveal test area are not dissimilar for the two subjects, but the curves for a 5° parafoveal test area are in sharp contrast. Whereas the 5° curve for H. R. S. approaches a final threshold value not very different from that of the 14° curve, the 5° curve for B. H. C. ends at a threshold value seven times that at 14°. It would seem that at 5° from the fovea H. R. S. has already reached a fairly constant type of parafoveal vision, while B. H. C. is still in an intermediate stage at this point. Confirmatory evidence of this appears in the next section 4·11.

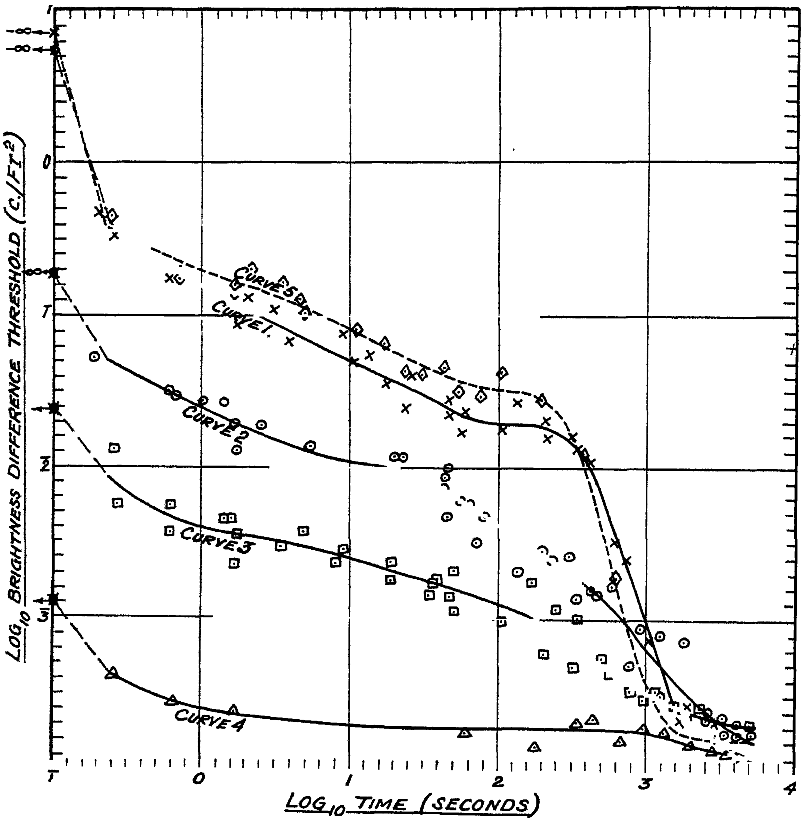

FIG. 6—Variation of brightness threshold with time after changing from various background brightnesses (B_0) to zero for a test-spot falling 14° from the fovea. Subject B. H. C., curve 1, $B_0 = 79$ c./ft.²; curve 2, $B_0 = 1{\cdot}59$ c./ft.²; curve 3, $B_0 = 0{\cdot}043$ c./ft.²; curve 4, $B_0 = 0{\cdot}000178$ c./ft.². Subject H. R. S., curve 5, $B_0 = 79$ c./ft.².

4·11—*Applications of the Equivalent Background Brightness Hypothesis*

It is possible to transform the results given in the preceding section by substituting for values of brightness threshold the brightnesses of the uniform background which would give the same values of brightness difference threshold under static conditions. The curves of variation of brightness threshold with time then become curves of variation of equivalent background brightness with time. The necessary accessory curves of brightness difference threshold against background brightness are given in fig. 7 for subjects B. H. C. and H. R. S. These curves give additional

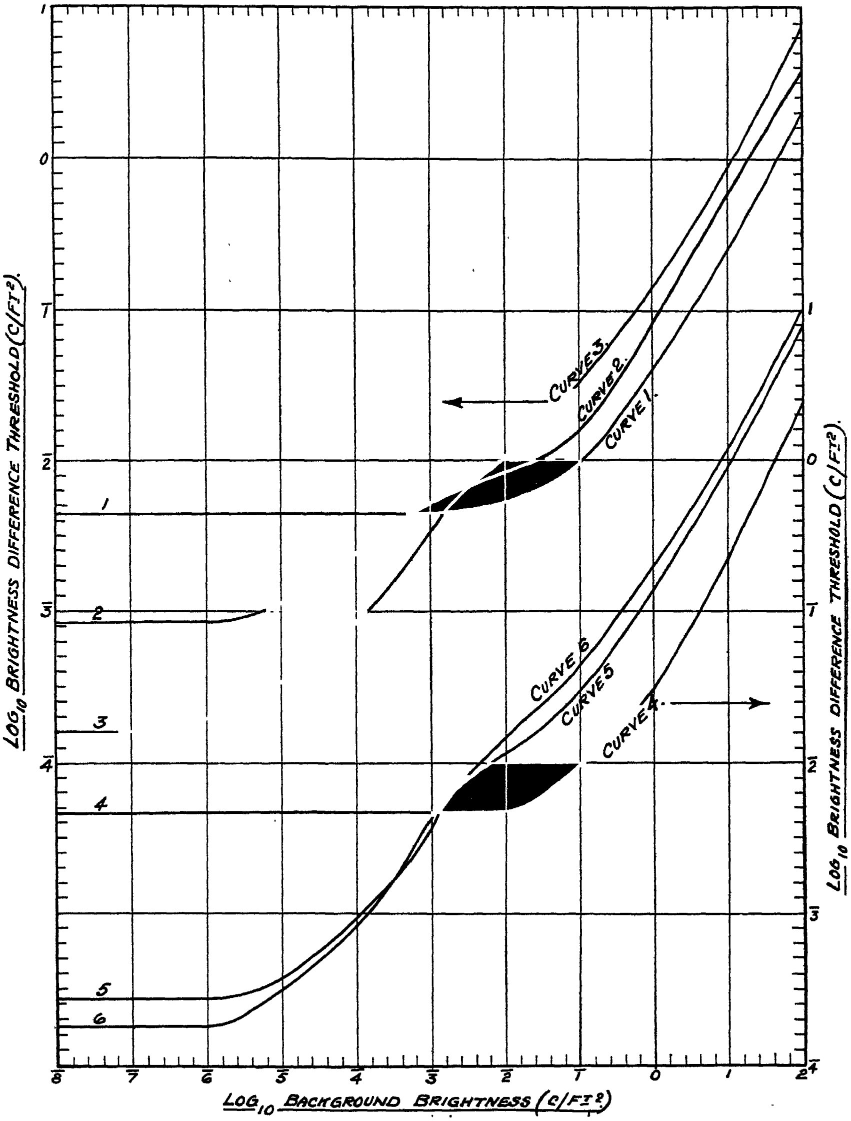

FIG. 7—Variation of brightness difference threshold with background brightness. Subject B. H. C., curve 1, fovea; curve 2, 5° parafovea; curve 3, 14° parafovea. Subject H. R. S., curve 4, fovea; curve 5, 5° parafovea; curve 6, 14° parafovea.

demonstration, as mentioned in the previous section, of the similarity in retinal response of B. H. C. and H. R. S. for foveal and for 14° parafoveal test areas, and of their marked dissimilarity for the 5° parafoveal test area. For H. R. S. the 5° parafoveal curve approaches closely to the 14°

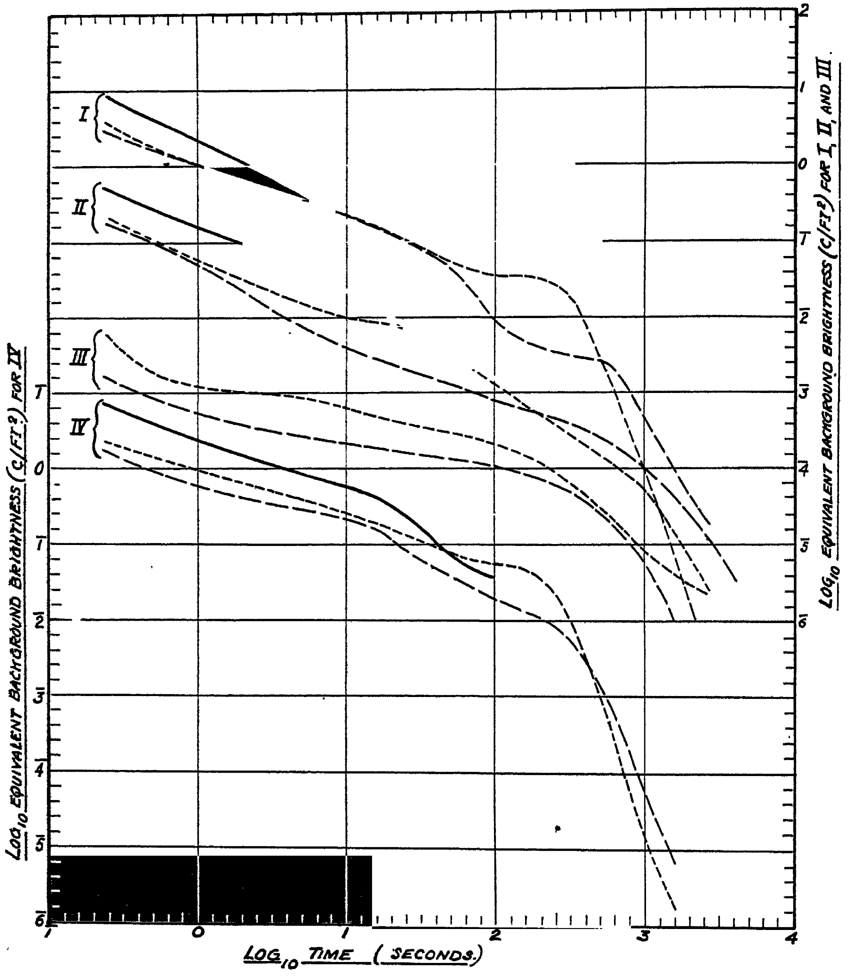

FIG. 8—Variation of equivalent background brightness with time after changing from various background brightnesses (B_0) to zero. Subject B. H. C., set I, $B_0 = 79$ c./ft.^{2}; set II, $B_0 = 1{\cdot}48$ c./ft.^{2}; set III, $B_0 = 0{\cdot}044$ c./ft.^{2}. Subject H. R. S., set IV, $B_0 = 79$ c./ft.^{2}. In all sets, fovea ——, 5° parafovea — —, 14° parafovea- - - -.

parafoveal curve, while for B. H. C. the 5° parafoveal curve lies about midway between the foveal and 14° parafoveal curves.

The effect of applying this transformation to the results of figs. 4–6 is shown in fig. 8. The point of interest is whether the various threshold curves for different retinal areas and the same initial background brightness will coalesce when transformed to equivalent background curves. Evidently they do not quite do this and the deviation between the curves is probably real, as both subjects show deviations in the same sense. From the point of view of practical applications, however, sufficient accuracy for most purposes would be attained by the use of a single (equivalent background brightness, time) curve for all retinal test areas. Also, comparison of the curves of set IV, fig. 8, subject H. R. S. with those of set I, subject B. H. C., shows that in both cases a very similar course is followed. It is possible that this might hold for all subjects, at least to a sufficient degree of accuracy for many practical applications. These equivalent background curves enable comparison to be made with earlier results (Stiles and Crawford 1932). It will be seen that the curves for the same primary conditioning stimulus are in approximate agreement with each other, the agreement being poorest for the longer times where the curves approach indeterminacy owing to lack of further variation in threshold.

The curves of fig. 8 may be used to test further a hypothesis regarding the mechanism of vision put forward in an earlier paper (Stiles and Crawford 1932). The results now available for several different primary conditioning stimuli do not support the formula deduced from the hypothesis, the deviations being quite outside any possible experimental error. An alternative hypothesis has not yet been formulated.

4·2—*Variation of Brightness Threshold after Changing from a Higher to a Lower Background Brightness*

The interesting point under this heading is the comparison of curves of the time variation of threshold taken with a fixed primary conditioning stimulus and a series of lower secondary stimuli down to zero. Results are shown in fig. 9. One subject only was used as the results turned out to be simple and unambiguous. It will be seen that, to a very fair degree of approximation, each curve follows the curve for a secondary stimulus of zero until the threshold value is reached which corresponds to the given secondary stimulus exposed steadily. The curve then flattens out and remains constant. There is some indication of the commencement of flattening out before this ideal point is reached, but the deviation is small and could be neglected for practical applications. Each curve is the mean of two complete sets of data and was drawn through the experimental points before being incorporated in the common diagram.

4·3—*Comparisons of (Brightness Threshold, Time) Variations using Different Types of Primary Conditioning Stimuli*

For these comparisons two different types of primary stimulus were used, a uniform background and a glare source. In each comparison the two primary stimuli were adjusted to give the same initial brightness threshold value (that is, during steady exposure of the primary stimulus) and the secondary stimulus was zero. The curves start at the same point and end at the same point: do they follow the same course in between? Within the limits of experimental error and over the range of times investigated they do, as is shown by fig. 10. The actual experimental points are plotted in this case, as giving the clearest demonstration of the question at issue and the degree of accuracy attained in its solution. Similar results were obtained for subject H. R. S., foveal test area.

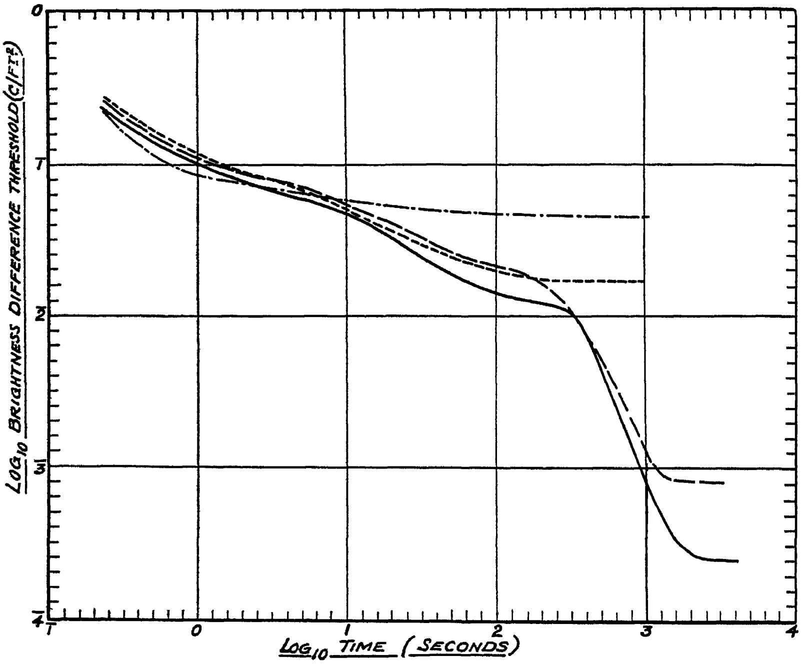

FIG. 9—Variation of brightness threshold with time after changing from one background brightness (B_0) to a lower brightness (B_1). Subject H. R. S., $B_0 = 81$ c./ft.². $B_1 = 0{\cdot}186$ c./ft.² — — —; $B_1 = 0{\cdot}0151$ c./ft.² - - - - -; $B_1 = 0{\cdot}000126$ c./ft.² — —; $B_1 = 0$ c./ft.² ——.

The backgrounds and glare sources used in the above comparisons are equivalent in the sense used by Holladay (1926) and later workers (Stiles

1929). That is, they produce at a certain specified test area of the retina the same value of some visual test when either is exposed steadily. The results now given indicate that they are also equivalent in the after effects which they produce. Hence the results of §§ 4·1 and 4·2 may be transformed to apply to a glare source as soon as one has determined the equivalent values of glare source and background by a straightforward static experiment. This leads to a very considerable reduction of the labour involved in obtaining a reasonably complete set of data. For many purposes, indeed, the procedure can be still further curtailed by making use

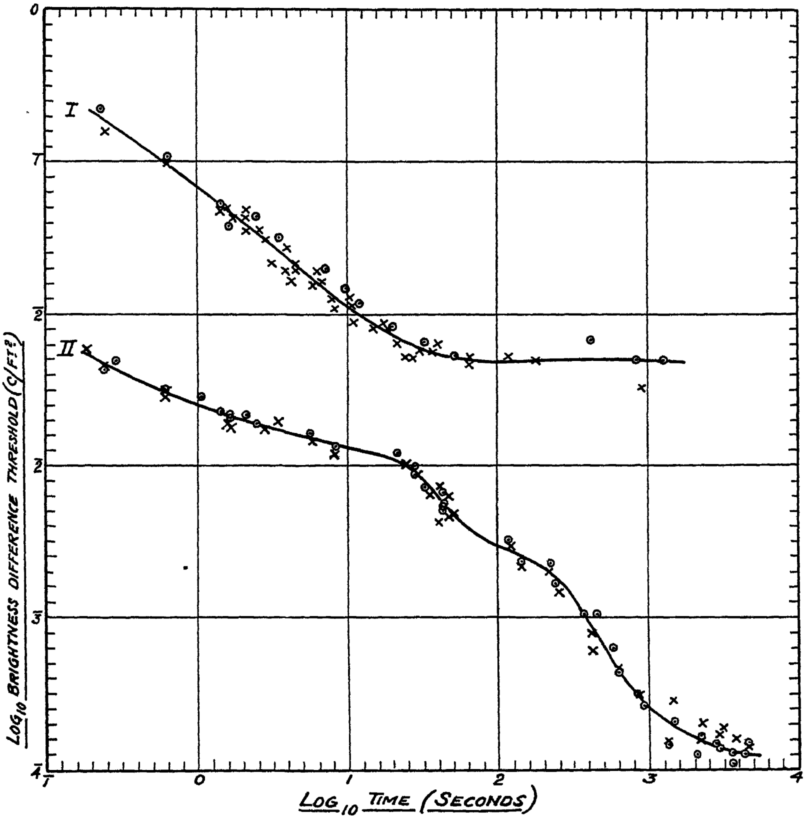

FIG. 10—Comparison of threshold variation with time after preliminary stimulation by a glare source and uniform background. Set I subject B. H. C., fovea; ×, glare source at 2·9° from fovea giving eye illumination of 65 c./ft²; ⊙, uniform background of brightness 71 c./ft.². Set II: subject B. H. C., 14° parafovea; ×, glare source at 10° from fovea giving eye illumination of 51 ft.c.; ⊙, uniform background of brightness 2·34 c./ft.².

of a formula established by several workers (Holladay 1926; Stiles 1929; Crawford and Stiles 1935):

$$\beta = B + \Sigma \frac{kE}{\theta^n}.$$

β is the equivalent background brightness, B is the actual background brightness, if any, E is the intensity of illumination at the subject's eye due to a glare source at angle $\theta°$ from the test area, k and n are constants, and Σ indicates summation of effects for any number of glare sources. The constants of the formula have been variously given:

$$\left.\begin{array}{l} k=9{\cdot}2 \\ n=2 \end{array}\right\} \text{Holladay} \quad \left.\begin{array}{l} k=4{\cdot}16 \\ n=1{\cdot}5 \end{array}\right\} \text{Stiles} \quad \left.\begin{array}{l} k=11{\cdot}5 \\ n=2{\cdot}1 \end{array}\right\} \text{Crawford and Stiles.}$$

Probably the figures $k=10$, $n=2$ are sufficiently near the truth for practical applications. These figures are also supported by further work by Stiles and Crawford (1937).

4·4—*Comparison of Different Varieties of Test-Spot*

Variations in size and time of exposure of the test-spot lead to variations in threshold brightness and so to various courses for the corresponding curves of threshold brightness against time. In view of the fact that different test-spots give also different curves of threshold brightness against background brightness, it is possible that the curves of equivalent background brightness against time would all follow the one course whatever the test-spot. This possibility has been explored by two subjects using three different test-spots, namely, 0·46° diameter and 0·05 sec. exposure, 0·46° diameter and 0·4 sec. exposure, 0·07° diameter and 0·4 sec. exposure; a round test-spot in each case. The results for one subject, B. H. C., are shown in figs. 11 and 12. Fig. 11 shows the variation of $\log_{10}$ brightness threshold with $\log_{10}$ time in the three lower curves. The three upper curves show the results in terms of equivalent background brightness, the transformation being made by means of the curves in fig. 12. Similar data have been obtained for subject H. R. S. The three equivalent background curves come into approximate coincidence in each case, and for calculations for practical purposes actual coincidence could be assumed. It may be pointed out, however, that the two subjects agree in making the curve for the 0·46°, 0·05 sec. test-spot run below the other two for times up to about 100 sec. The difference is not great, but it would be desirable by further data to substantiate or eliminate it in view of theoretical consequences.

In conclusion, the author would express his great appreciation of the assistance given by Mr. H. R. Sayer in carrying out the experimental work

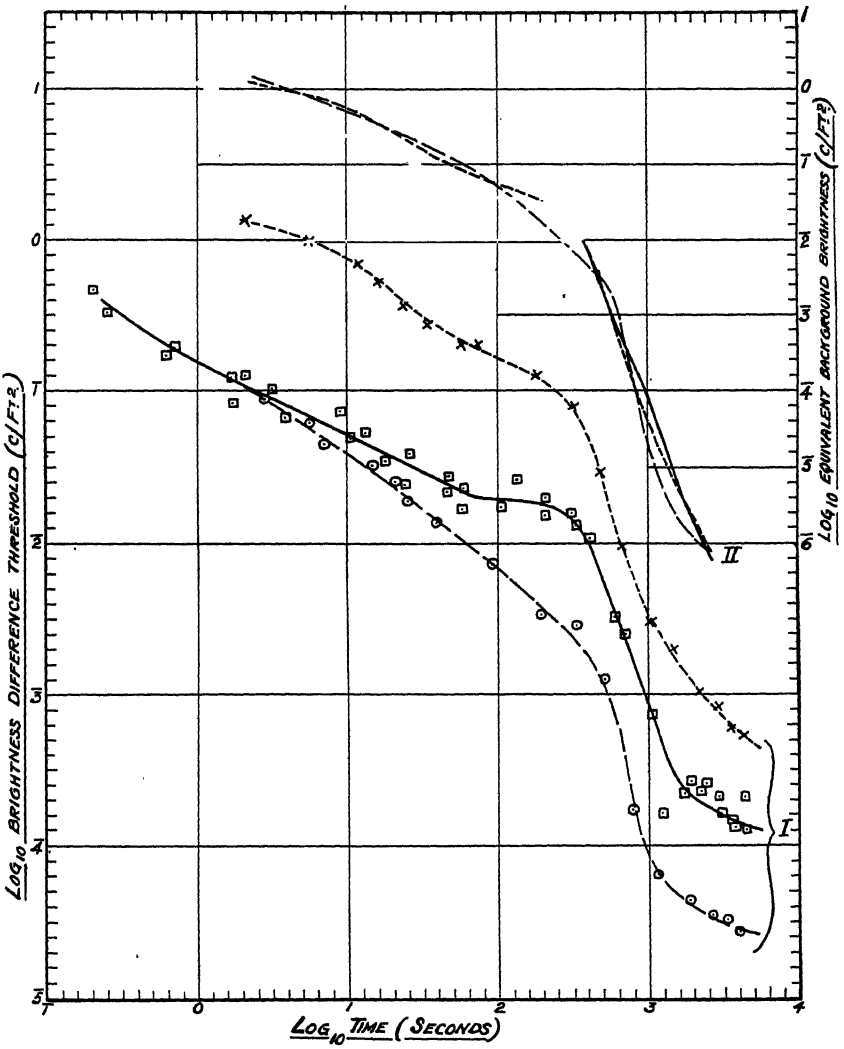

FIG. 11—Variation of brightness threshold (I) and of equivalent background brightness (II) with time after cutting off an initial background brightness of 79 c./ft.² using various test-spots: 0·46° diameter, 0·05 sec. exposure ——; 0·46° diameter, 0·4 sec. exposure — —; 0·07° diameter, 0·4 sec. exposure - - - - -; subject B. H. C., 14° parafovea.

and in taking the observations, and of the advice and criticism of Dr. W. S. Stiles during the planning and course of the work, which was carried out under the auspices of the Illumination Research Committee of the Department of Scientific and Industrial Research.

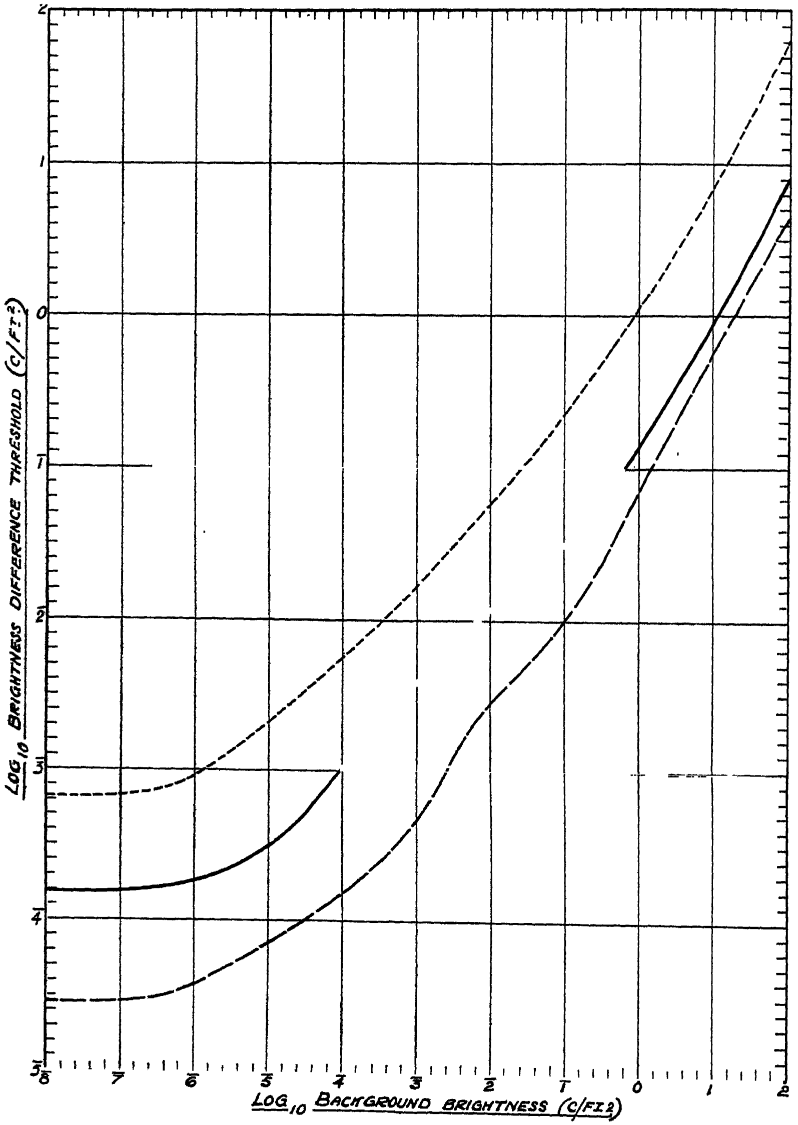

FIG. 12—Variation of brightness difference threshold with background brightness for various test-spots; 0·46° diameter, 0·05 sec. exposure ——; 0·46° diameter, 0·4 sec. exposure — —; 0·07° diameter, 0·4 sec. exposure - - - - -. Subject B. H. C., 14° parafovea.

SUMMARY

1. Results are given showing the variation of visual sensitivity after cutting off a high background brightness, the measure of sensitivity being the least brightness visible to the eye. Small retinal areas at the fovea, 5° from the fovea and 14° from the fovea were used for sensitivity measurements. Expression of the results in terms of equivalent background brightness enables considerable simplifications to be made for practical applications of the results.

2. The variation of brightness threshold after changing from a high to a low background brightness is investigated and shown to bear a simple relation to the case of changing from a high to zero background brightness.

3. Comparison is made of the sensitivity variations after cutting off a uniform background and a punctiform glare source, both of which initially gave the same sensitivity. The results indicate that, over the range tested, the pattern of the initial stimulus is without influence on the subsequent variation of sensitivity.

4. Three test-spots differing in size and time of exposure are compared and shown to give approximately the same curves of sensitivity variation with time when the actual threshold values are replaced by their equivalent background brightnesses.

In all sections, the test-spot and primary and secondary conditioning stimuli were white.

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The Luminous Efficiency of Monochromatic Rays Entering the Eye Pupil at Different Points and a New Colour Effect

By W. S. Stiles

From The National Physical Laboratory

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Introduction

It has been found by the writer, in collaboration with B. H. Crawford (1933), that light rays entering the eye pupil near its periphery are less efficient in producing the impression of brightness than rays entering centrally, the patch of retina stimulated (the fovea) being the same in both cases. Reasons were put forward in the paper cited for thinking the effect to be retinal in origin, i.e. due to a variation of visual sensitivity with angle of incidence of the light on the retina, rather than the result of a greater absorption of the peripheral rays in transit through the optic media of the eye. Most of the observations were made with white light and, although the absence of any pronounced coloration of the field illuminated by the peripheral ray indicated that the reduction of apparent brightness could not be very different for different colours, it was considered desirable to test this point directly by observations with monochromatic light throughout the spectrum. In Part I of this paper an investigation on these lines is described from which it appears that for the writer's eye the ratio of the luminous efficiencies of rays entering centrally and peripherally varies systematically to a limited extent in passing through the spectrum. It was also found that within a considerable range of intensity the value of the ratio for a given wave-length is independent of intensity.

Since the publication of the original paper, Dziobek (1934) and Wright and Nelson (1936) have both made measurements confirming the existence of a marked variation of luminous efficiency with point of entry. The latter workers employed white light and coloured lights obtained with the aid of filters. Goodeve (1936) has also measured the effect, in the extreme red.

In making measurements with monochromatic light it was observed that as the point of entry of the light ray moved across the pupil, the corresponding half of the photometric matching field exhibited in some parts of the spectrum a change of colour in addition to the change of brightness. This colour change, which as far as is known has not previously been recorded, is of particular interest as it provides evidence of a difference in properties of the three types or groups of types of receptor postulated in the trichromatic theory other than differences in their spectral excitation curves. Part II of the paper gives an account of measurements of the colour effect together with certain deductions from the results so far obtained.

PART I—THE EFFECT OF WAVE-LENGTH ON THE LUMINOUS EFFICIENCIES OF RAYS ENTERING THE EYE PUPIL AT DIFFERENT POINTS

Apparatus and Method

A diagram of the apparatus used is shown in fig. 1. The source S is a ribbon filament gas-filled tungsten lamp (6 V, 100 W) placed so that the length of the ribbon is horizontal and inclined at an angle of 45° to the axes of the two optical trains. Lens L_1 ($f = 17$ cm.) forms an image of S on the entrance slit of the Hilger constant deviation spectrometer I which has collimator and telescope lenses of 28·5 cm. focal length. In front of the exit slit of spectrometer I (here used as a monochromator) two gelatine neutral wedges W_1 and W_2 are arranged to move in vertical guides, W_1 having a continuous variation of density and W_2 a stepwise variation. The light from the exit slit of spectrometer I is rendered parallel by lens L_2 ($f = 24$ cm.); the beam is delimited by a square aperture in the diaphragm T_1 and, after passing through the glass cube C, is brought to a focus ω_1 approximately in the plane of the subject's eye pupil (at O) by the lens L_3 ($f = 24$ cm.). The cube C, of 5 cm. side, consists of two right-angled prisms enclosing between their opposed hypotenuse faces a thin half-platinized glass plate, the prisms and plate being cemented together with Canada balsam and firmly clamped in a brass frame.

In the second optical train the lenses L_6 ($f = 17$ cm.) and L_5 ($f = 25$ cm.) produce an image of S on the entrance slit of the Tutton monochromator spectrometer II which has collimator and telescope lenses of focal length 18·5 cm. A wedge W_3 having a continuous variation of density moves in vertical guides in front of the exit slit of spectrometer I. Lens L_4 ($f = 24$ cm.) and diaphragm T_2 delimit a small parallel beam of square section as in the first optical train. This beam is partially reflected at the half-platinized plate in the cube *C* and is brought to a focus ω_2 in the plane of the subject's eye pupil by the lens L_3.

The diaphragms T_1 and T_2 can be adjusted vertically and laterally so that the eye at *O* sees by Maxwellian view two juxtaposed square fields in contact along a horizontal edge and forming together a bipartite photometric matching field of total angular dimensions $1{\cdot}8 \times 0{\cdot}9^\circ$ (approx.). The diaphragms T_1 and T_2 are normally placed at such a distance from the cube *C* that they lie at the focus of lens L_3 (allowing for the presence of the glass cube) and are seen by the subject at infinity. Owing to the chromatic aberrations of the eye and residual chromatic aberrations of the optical system, it was found convenient to adjust the distances of the diaphragms T_1 and T_2 for each wave-length studied to give a field of optimum sharpness as seen by the subject with his eye as far as possible in the unaccommodated condition. It may be noted here that all the lenses used in the apparatus are achromatic doublets.

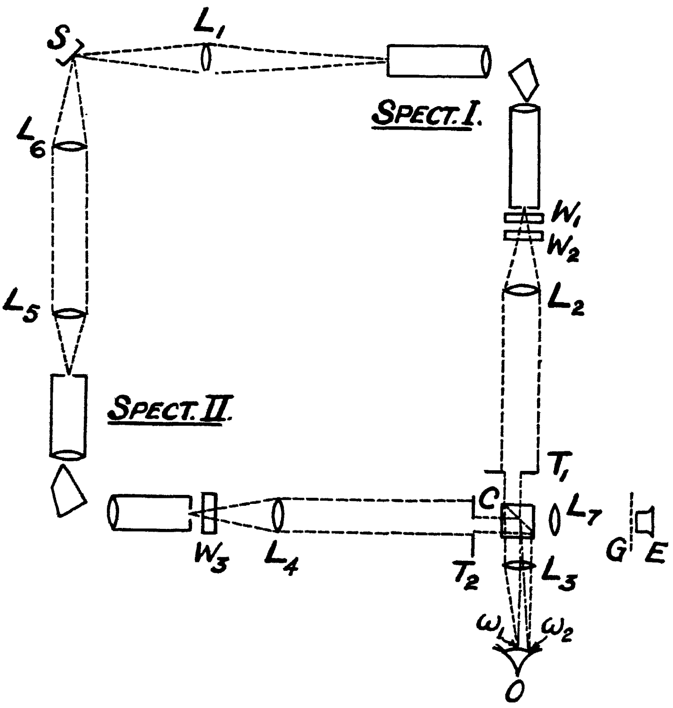

Fig. 1—Diagram of apparatus (not to scale).

The cube *C* is arranged to rotate about a vertical axis through its centre, the amount of rotation being controlled by means of a micrometer screw.

As the cube rotates, the image ω_2 moves on a horizontal line across the subject's pupil while the image ω_1 remains practically stationary. When such a rotation is made, the subject sees the two square fields separate laterally. This is due in the main to an actual change in the position of the virtual image of T_2 formed by reflexion in the half-platinized plate of the cube. There is in addition an apparent shift in the position of T_2 due to the spherical aberration of the subject's eye. The two fields can be brought back into position by a displacement of the diaphragm T_2. For determining the relative positions of ω_1 and ω_2 in the plane of the subject's pupil, an auxiliary optical system comprising the lens L_7 ($f = 24$ cm.), a graticule G at the focus of L_7 and an eye-piece E for viewing the graticule, is used. Images ω_1' and ω_2' of the exit slits of spectrometers I and II respectively are formed in the plane of the graticule, ω_1' by reflexion in the cube and ω_2' by transmission through the cube. When ω_1 and ω_2 are coincident, so are ω_1' and ω_2'. When C is rotated, ω_2 moves away from ω_1 and at the same time ω_1' moves away from ω_2' by a proportional amount. By setting up a travelling microscope to view ω_1 and ω_2 in the plane normally occupied by the subject's eye pupil, the displacement of ω_1' and ω_2' on the graticule corresponding to a given displacement of ω_1 and ω_2 in the eye plane can be determined.

In using the apparatus it is clearly essential for the observing eye to remain in a fixed position. The head is kept in a fixed position by making the subject bite on a sealing wax impression of his teeth carried on a brass plate fixed to a heavy metal frame which is itself clamped firmly to the table carrying the apparatus. When the head is in a fixed position and the gaze is directed to the centre of the matching field, it may be assumed that the subject's eye is also in a fixed position. The metal frame incorporates adjustments which enable the sealing wax "bit" to be displaced in three directions at right angles so that the centre of the subject's pupil can be brought into coincidence with the fixed image ω_1.

Principle of the Measurements

With the images ω_1 and ω_2 coincident and entering the subject's pupil at its centre, and with both spectrometers selecting light of the same wavelength λ, the subject adjusts the wedge W_3 until the two halves of the photometric field formed by the diaphragms T_1 and T_2 appear equally bright.

The setting is repeated several times. The experimenter then turns the micrometer screw to rotate C by a certain amount and notes the displacement of the image ω_1' on the graticule. The subject readjusts the diaphragm T_2 to restore the juxtaposition of the two fields and makes a new series of settings of the wedge W_3 to give equality of brightness between the two fields. If Δ is the density, for wave-length λ, corresponding to the initial mean setting of the wedge and if Δ_d is the density for the mean setting when ω_2 is displaced d mm. from the pupil centre, then the luminous efficiency η for the displaced position of entry of the light is given by

$$\log_{10}\eta = \Delta_d - \Delta.$$

Continuing the measurements in the above manner the whole diameter of the pupil can be traversed and a curve drawn relating $\log_{10}\eta$ to d.

(In the previous paper η and not $\log_{10}\eta$ was plotted against d. The present procedure has some advantages.)

Spectrometer Adjustments, etc.

The focusing rings on the spectrometers were calibrated to enable the image of the entrance slit to be brought into focus in the plane of the exit slit at each wave-length. A wave-length calibration curve was used for spectrometer I but was unnecessary for spectrometer II. The telescope and collimator of spectrometer I were each equipped with a microscope interchangeable objective holder (Beck) modified to take fixed slits in place of objectives. The device enabled slits to be removed and then replaced in exactly the same position. For the exit slit a fixed slit of width 0·48 mm. and height 0·50 mm. was used throughout the measurements. For the entrance slit, fixed slits of height 2 mm. and widths 0·23, 0·45, 1·06 and 2·01 mm. respectively were used as required. Spectrometer II was fitted with symmetrically opening micrometer slits supplied with the instrument (Hilger). The slit height could be controlled by the V-shaped slides provided but with the small height of exit slit here required the irregularities at the apex of the V gave trouble. The slits were therefore delimited vertically by adjustable bevelled brass strips screwed to the V slides. For the entrance and exit slits, heights of 0·32 and 1 mm. respectively were used for most of the measurements.

The difference of wave-length $\Delta\lambda$ between the extreme limits of the spectrum band passed by the spectrometer may be used to specify the nominal purity of the light if the slit widths are equal. When this is not the case it is necessary to state in addition the ratio ρ of the exit and entrance slit widths. The actual purity obtained will depend on the amount of light scattered or irregularly reflected in the spectrometer. The parasitic light arising in this way was reduced to a point at which it could have little, if any, effect on the measurements by inserting suitably chosen coloured glass filters in the optical system between the source and the entrance slits of the spectrometers. The effectiveness of the glasses used was checked qualitatively by letting the images ω_1 and ω_2 fall on the entrance slit of a direct vision spectrometer and observing the spectrum produced.

Calibration of the Wedges

The wedges W_1, W_2 and W_3 were calibrated in the apparatus by the usual photometric methods, employing a series of rotating sectors of known transmissions. The photometric matches for these measurements were made of course with the images ω_1 and ω_2 coincident and entering the eye centrally. With a sector of density Δ (density $= \log_{10}(1/\text{transmission})$) inserted between L_5 and the entrance slit of spectrometer II and with wedge W_3 raised out of the beam emerging from the exit slit, wedge W_1 was adjusted to give a photometric match between T_1 and T_2. Leaving this setting of W_1 unchanged, the sector disk was removed and wedge W_3 lowered into the beam and adjusted to restore the photometric match. The reading r on the vertical scale of the index mark carried by W_3 is then known to correspond to a wedge density Δ. The calibration curve relating Δ and r was determined for W_3 for light of wave-length $\lambda_0 = 540\,\text{m}\mu$. The range of density equalled approximately 2·5 and the calibration curve was nearly, but not quite, rectilinear. Further measurements showed that for any other wave-length λ, the wedge density Δ was related to the density Δ_0 for λ_0, by the equation

$$\Delta = a + b\Delta_0,$$

where a and b are constants depending on wave-length but approximately independent of density. Fig. 2 shows the variation of a and b with wave-length. The two curves of fig. 2 together with the calibration curve for $\lambda = 540\,\text{m}\mu$ provide a complete calibration of the wedge W_3 for light of any wave-length. Wedges W_1 and W_2 were calibrated similarly although their calibrations were not required for the main measurements.

Sizes of Images ω_1 and ω_2, Centring of the Eye, etc.

The optical system is such that the images ω_1 and ω_2 are of the same size as the corresponding exit slits. Their vertical dimensions were therefore 0·50 mm. (ω_1) and 0·32 mm. (ω_2) approximately and the width of ω_1, 0·48 mm. The width of ω_2 depended on the wave-length used but never exceeded 0·50 mm. The accurate centring of the eye was accomplished by setting ω_2 and ω_1 to be coincident and traversing the subject's head in a vertical or horizontal direction with the aid of the movements on the bit-holder until the images ω_1 and ω_2 crossed the edge of the dilated pupil.

This was apparent by the sudden extinction of the light in the fields T_1 and T_2 as seen by the subject. The midpoint of the two settings for extinction gave the setting for central entry. A mydriatic (euphthalmine) was used to obtain a steady and fully dilated pupil. The subject sat in a small enclosure formed of black curtains and the idle eye was covered with an eye shade. An aperture in the front curtain disclosed the lens L_3. Thus the field was dark save for the two fields to be matched.

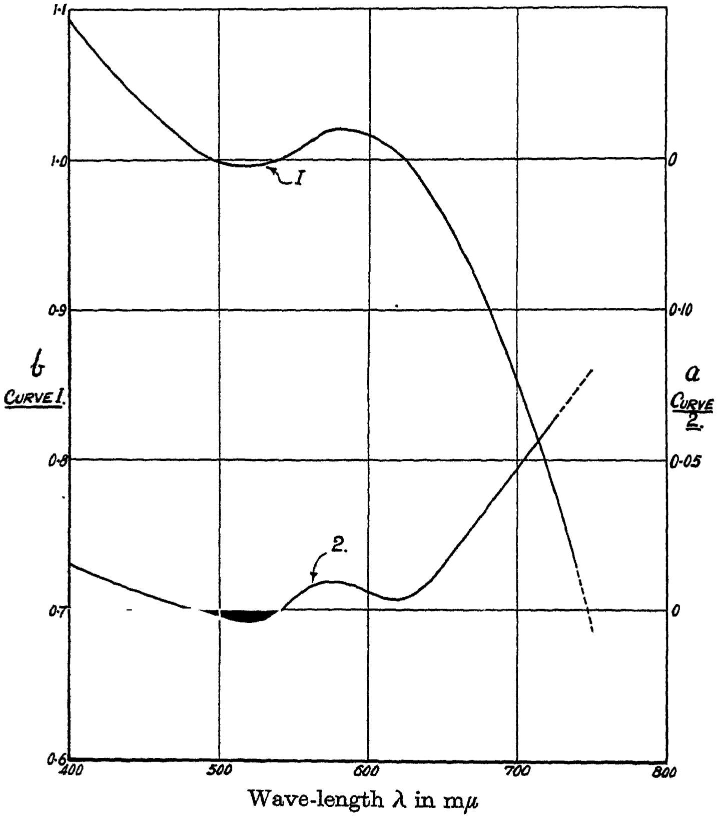

FIG. 2—Wave-length corrections to density calibration curve of wedge W_3.

Possible Errors due to the Adjustment of T_2

When the image ω_2 is traversed across the eye horizontally it is necessary to move the diaphragm T_2 slightly to keep the matching fields juxtaposed. This means that a slightly different portion of the parallel beam issuing from the lens L_4 is selected by the diaphragm and a slightly different part of the half-platinized plate is employed in the reflexion. Estimates of the possible errors arising in this way from non-uniformity in the beam and in the half-platinized plate showed them to be small and probably not greater than about 5 % in the worst case.

Absolute Intensities

Although the measurements were primarily concerned with the change in apparent brightness with change in the point of entry of the ray in the eye pupil, it was desirable to know, at least approximately, the absolute intensity of the matching field. Suppose θ° K. is the colour temperature of the source S when run at a given voltage. We can determine from tables such as those of Skogland (1929) the radiational energy in the wave-length interval λ to $\lambda + d\lambda$ emitted per sec. in all directions by 1 sq. cm. of a black body at temperature θ. Putting this equal to $J(\theta,\lambda)\,d\lambda$, the corresponding quantity for a tungsten emitting surface will be $\epsilon(\theta).J(\theta,\lambda)\,d\lambda$, where $\epsilon(\theta)$ is an empirical constant termed the colour emissivity of tungsten at the temperature (θ) (Forsythe and Worthing 1926).

It can now be shown without difficulty that the total energy flux in eyes per sec. incident on the eye at O from that part of the photometric field illuminated by spectrometer I is equal to

$$\frac{\epsilon(\theta)}{\pi}.J(\theta,\lambda)\,h\sigma_1\sigma_2S_\lambda t_\lambda\tau_\lambda\Phi, \qquad \text{......(1)}$$

where

σ_1 = width in cm. of entrance slit of spectrometer I.

σ_2 = width in cm. of exit slit of spectrometer I.

h = height in cm. of entrance or exit slit of spectrometer I whichever is the lesser. In our case the exit slit has the lesser height.

S_λ = dispersion of spectrometer I expressed as the width in microns of the band of spectrum passed per cm. width of exit slit, when the entrance slit is a mathematical line.

t_λ = overall transmission for wave-length λ of all the optical parts between light source and eye including the transmission of any stray light glass which may be used.

τ_λ = transmission for wave-length λ of the wedges W_1 and W_2 at any particular settings.

Φ = solid angle in steradians subtended at the eye by that part of the photometric field illuminated by spectrometer I.

It is more appropriate to express the intensity as the energy flux incident on the eye per unit solid angle of the field, and it is also convenient to express solid angle in square degrees rather than in steradians. Thus we shall put

U_λ equal to the energy flux incident on the eye expressed in ergs per sec. per square degree of the field. U_λ is given simply by the expression (1) above with the omission of the factor Φ and the introduction of a constant factor $(1/57{\cdot}3)^2$ which takes account of the use of square degrees in place of steradians. A closely similar expression to (1) is obtained for the intensity in energy units U_λ of the part of the field illuminated by spectrometer II.

An accurate determination of the absolute field intensity was not required and the values of U_λ quoted in what follows must be regarded only as very approximate.

In addition to U_λ, the photometric brightness B of the field, defined in the following conventional manner, will also be given. B is equal to the brightness in candles per square which, if viewed through an artificial pupil of 10 sq. mm. area by an eye possessing the standard relative luminosity curve, would match in brightness the monochromatic field as seen in the apparatus. Actually the existence of the pupil effect calls for certain refinements in this definition, but for the present purpose these may be neglected. It is then easy to show that B equals

$$U_\lambda V_\lambda \frac{57{\cdot}3^2}{15000 \times 1{\cdot}08 \times 10^{-5} \times 10} = 2{\cdot}02 \times 10^3 U_\lambda V_\lambda, \qquad \ldots\ldots(2)$$

where V_λ is relative luminosity factor for wave-length λ.

By arranging to illuminate one-half of the photometric field of the apparatus with a diffusely emitting white surface and by matching this surface seen through an artificial pupil with the other half of the photometric field illuminated by yellow light of wave-length 580 mμ from spectrometer I, a comparison was made between the brightness of the yellow half field obtained by calculation from the expressions (1) and (2) and the brightness of the white half field obtained by direct measurement with an illuminometer set up in the eye position (the artificial pupil was removed for this purpose). The values obtained differed by 26 %, which is not a large discrepancy bearing in mind the approximations made.

Results

Fig. 3 shows the variation of $\log_{10}\eta$ with d for two horizontal traverses through the centre of the pupil using light of wave-length 500 mμ. Each plotted point is the mean of four settings. The circles represent observations obtained in a traverse in the direction nasal to temporal, the crosses observations obtained in a second traverse in the reverse direction (additional points at $d = 0$ are shown as squares). Similar sets of data were obtained for other wave-lengths ranging from $\lambda = 440$ to $\lambda = 720$ mμ, the details of these runs being given in Table I. In each case a continuous curve was drawn through the plotted points as shown in fig. 3. The data for $\lambda = 500\,\mathrm{m}\mu$ are among the best in showing agreement between the observations for the

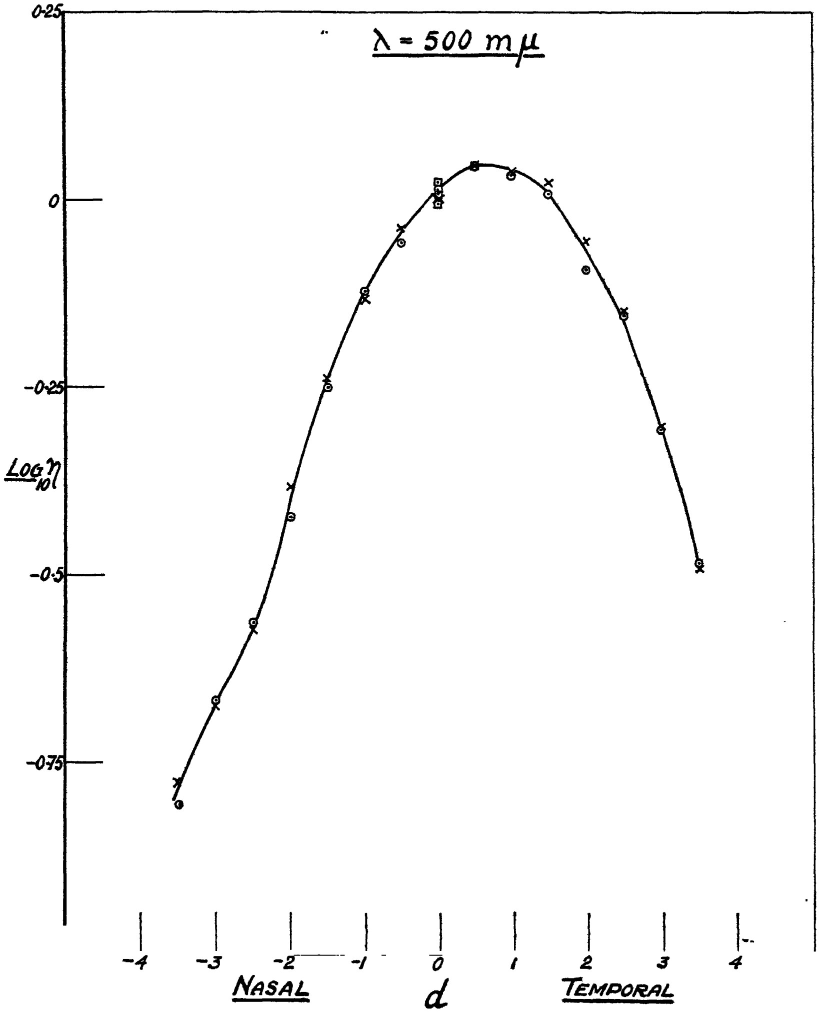

FIG. 3

two traverses. All the results are for the author's left eye dilated with euphthalmine.

As anticipated, the general form of the curve of $\log_{10}\eta$ against d is not very different for different wave-lengths. However, an analysis of the data on

Table I—Values of $\log_{10}\eta$ for Different Wave-lengths obtained in Complete Horizontal Traverses of the Pupil. Subject W. S. S. Left Eye. Pupil dilated with Euphthalmine

λ (mμ)	440	460	480	500	520	540	560	580	600	620	660	720	
$\Delta\lambda$ (mμ)	9·6	10·1	10·0	9·8	10·2	10·0	10·0	10·3	10·0	10·0	10·0	25·0	
ρ	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	
$\log_{10} U_\lambda$	$\bar{4}$·0	$\bar{4}$·0	$\bar{4}$·4	$\bar{4}$·6	$\bar{4}$·5	$\bar{4}$·3	$\bar{4}$·9	$\bar{4}$·8	$\bar{4}$·8	$\bar{4}$·8	$\bar{4}$·9	$\bar{3}$·7	
$\log_{10} B$	$\bar{3}$·6	$\bar{2}$·1	$\bar{2}$·8	$\bar{1}$·4	$\bar{1}$·6	$\bar{1}$·6	0·2	0·0	$\bar{1}$·9	$\bar{1}$·7	$\bar{1}$·0	$\bar{2}$·0	
d (mm.)													Mean data
−3·5 nasal	0·97	0·86	0·86	0·79	0·70	0·69	0·73	0·72	0·74	0·74	0·71	0·73	0·770
−3·0 ,,	0·79	0·82	0·76	0·67	0·60	0·52	0·60	0·60	0·60	0·66	0·62	0·61	0·655
−2·5 ,,	0·60	0·66	0·62	0·57	0·44	0·41	0·43	0·49	0·47	0·52	0·50	0·50	0·519
−2·0 ,,	0·44	0·47	0·46	0·40	0·28	0·30	0·33	0·36	0·31	0·37	0·36	0·39	0·373
−1·5 ,,	0·26	0·27	0·27	0·24	0·17	0·18	0·21	0·24	0·22	0·24	0·23	0·22	0·231
−1·0 ,,	0·15	0·17	0·12	0·13	0·08	0·10	0·12	0·11	0·13	0·14	0·11	0·08	0·120
−0·5 ,,	0·05	0·07	0·05	0·05	0·05	0·05	0·06	0·02	0·04	0·08	0·04	0·02	0·050
0 ,,	0	0	0·01	0	0	0	0	0	0	0·02	0·01	0	0·004
0·5 temporal	−0·01	−0·04	−0·03	−0·05	0	0·01	−0·02	−0·04	−0·02	0·01	−0·05	−0·02	−0·018
1·0 ,,	0·01	0	−0·03	−0·03	0·03	0·04	−0·02	−0·03	−0·01	−0·01	−0·03	−0·02	−0·009
1·5 ,,	0·06	0·02	0	−0·02	0·06	0·08	0·03	0·02	0·01	0·02	0	−0·01	0·023
2·0 ,,	0·13	0·05	0·05	0·07	0·13	0·18	0·12	0·10	0·10	0·12	0·07	0·02	0·095
2·5 ,,	0·28	0·19	0·17	0·15	0·25	0·24	0·26	0·20	0·19	0·25	0·23	0·22	0·219
3·0 ,,	0·46	0·37	0·35	0·30	0·39	0·38	0·40	0·39	0·37	0·42	0·39	0·35	0·381
3·5 ,,	0·61	0·53	0·50	0·49	0·48	0·57	0·50	0·43	0·42	0·53	0·48	0·50	0·503
p	0·0721	0·0712	0·0688	0·0627	0·0532	0·0538	0·0567	0·0595	0·0540	0·0604	0·0639	0·0618	0·0612
d_m (mm.)	0·49	0·65	0·69	0·68	0·35	0·28	0·50	0·57	0·57	0·58	0·59	0·59	0·56

the following lines reveals a small systematic variation through the spectrum.

The mean value of $\log_{10}\eta$ for all wave-lengths at each *d* value was first determined, and the mean curve so obtained is shown as the continuous curve on fig. 4. (Mean data of Table I.) The curve is nearly, but not quite, symmetrical about an ordinate displaced 0·6 mm. to the temporal side. Thus the luminous efficiency is a maximum for rays entering slightly to the temporal side of the pupil centre.

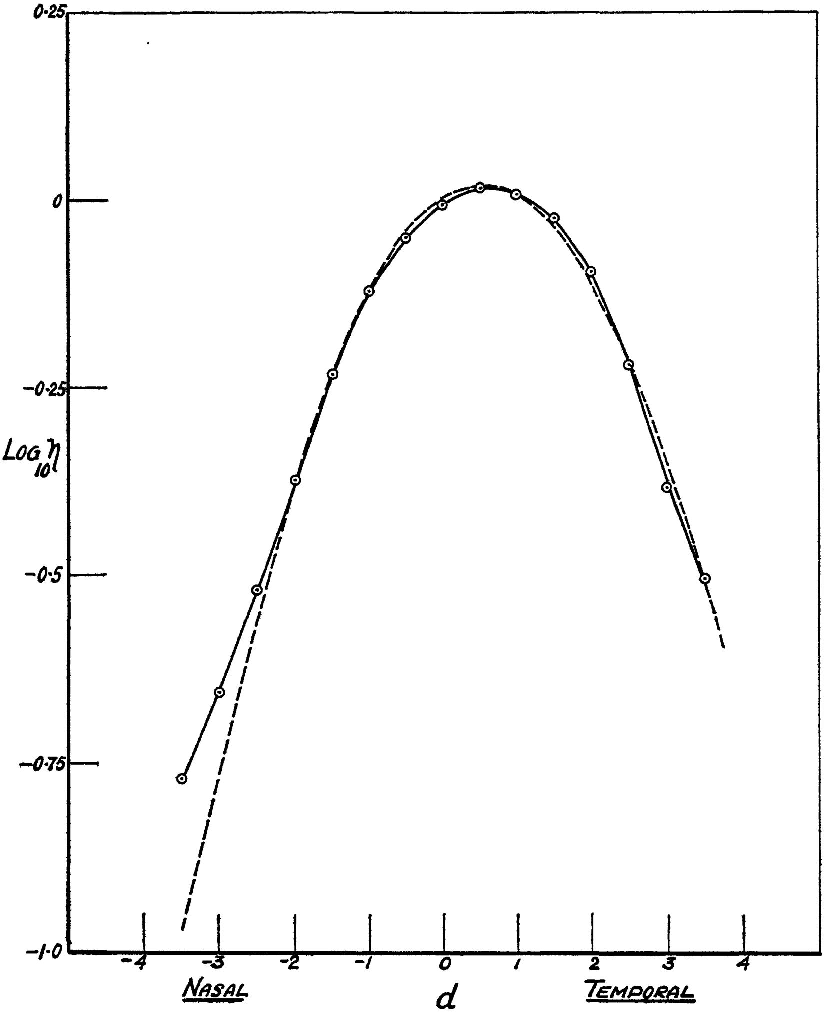

Fig. 4—Mean curve for all wave-lengths.

The broken curve shown in fig. 4 is represented by the formula:

$$\log_{10}\eta - \log_{10}\eta_m = -p\,(d-d_m)^2, \qquad \ldots\ldots(3)$$

with $p = 0{\cdot}0612$, $d_m = +0{\cdot}56$ mm., where η_m is the value of η at $d = d_m$. The broken curve and the observed mean curve are in tolerably good agreement for values of $(d-d_m)$ not greater than about 3 mm. When $(d-d_m)$ exceeds 3 mm. (only obtainable on the nasal side) the observed effect is less than that indicated by the formula.

It was found that a formula of the type (3) could be used to represent the curves of $\log_{10}\eta$ against d for the individual wave-lengths with about the same measure of agreement as for the mean curve. The values of the constants p and d_m for the different wave-lengths are shown in the lowest rows of Table I. They were obtained by fitting to the data over the range $d = -2\frac{1}{2}$ to $d = +3\frac{1}{2}$ mm. Considering first the values of d_m, which represents the displacement of the maximum of the fitted curve from the pupil centre, it is found that, excluding the values for $\lambda = 520\,\mathrm{m}\mu$ and $\lambda = 540\,\mathrm{m}\mu$, d_m differs from the mean value by not more than 0·1 mm. Such a range of variation is of the order to be expected from errors in determining the pupil centre and in fitting the empirical formula. For the two wave-lengths mentioned, d_m differs from the mean of the values for the remaining wave-lengths by 0·27 and 0·31 mm. respectively. This discrepancy is too large to be readily accounted for by errors in determining the centre of the dilated pupil or in the curve fitting. However, we cannot be sure that the pupil, when treated with the mydriatic, always dilates in such a way that its centre occupies exactly the same position with respect to the rest of the eye. In later measurements, the position of the maximum in the $\log_{10}\eta$ curve was determined for the three wave-lengths, $\lambda = 480$, 530 and $600\,\mathrm{m}\mu$, on the same day without altering the adjustment of the "bit" or other parts of the apparatus. The values found differed from their mean by 0·06, 0·04 and 0·01 mm. respectively, deviations lying within the experimental error. It is highly probable therefore that the $\log_{10}\eta$ curve shows no bodily shift to one side or the other as the wave-length of the light is changed.

Turning to p, which may be regarded as specifying the magnitude of the effect being studied, the values for the different wave-lengths are plotted in fig. 5. It appears that the effect is greatest in the blue end of the spectrum, smallest in the green and intermediate in the red.

Since the curves on which fig. 5 is based were determined over a period of several weeks, it was desirable to make further measurements in which the complete range of wave-lengths was covered in a single run. In the previous runs the traversing image ω_2 was moved across on a horizontal line through the pupil centre and the greatest luminous efficiency for any point on this line was found to occur at about 0·5 or 0·6 mm. to the temporal side of the pupil centre. It does not follow, and for the author's left eye it is not the case, that the luminous efficiency at $d = 0{\cdot}5$ mm. is greater than for any other point of the pupil. By determining the luminous efficiency at different points on a *vertical* line 0·5 mm. to the temporal side of the pupil centre, it was found that the maximum efficiency occurred at a point 0·5 mm.

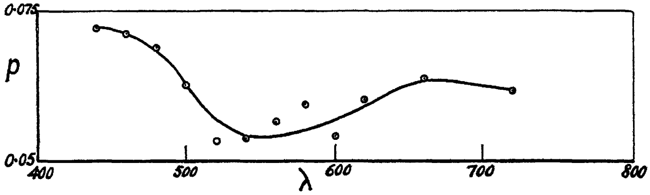

FIG. 5

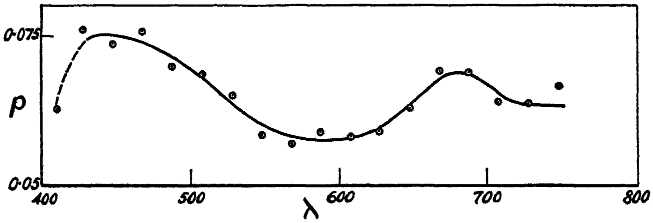

FIG. 6

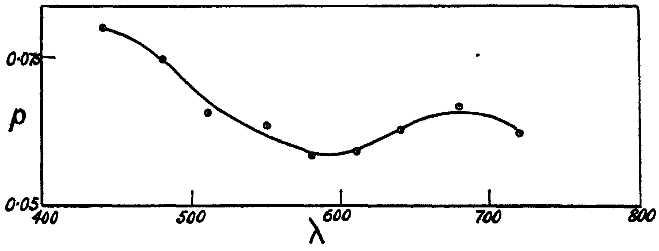

FIG. 7

above the horizontal through the pupil centre. This point, which will be referred to as P_M, corresponds approximately to the maximum luminous efficiency obtainable for any point of entry. In the measurements now to be recorded, the luminous efficiency, when the traversing beam (ω_2) entered at P_M, was compared with that obtained when it entered at a point Q, 2·75 mm. to the nasal side of P_M. The fixed comparison beam was arranged to enter at P_M for both observations. Measurements for all the wave-lengths studied were made without interruption in one run, the adjustment of the whole apparatus being kept unchanged save for varying the wave-length.

Assuming that a formula of the type (3) will apply for a horizontal traverse through the point P_M, it follows that

$$(\log_{10}\eta)_{P_M}-(\log_{10}\eta)_Q=-p(0-r_m)^2-\{-p(-2\cdot75-r_m)^2\}=p\{7\cdot56+5\cdot5r_m\},$$

where r_m is the displacement from the point P_M of the position in the traverse at which the luminous efficiency is a maximum. By our choice of P_M, r_m should be zero but owing to errors in determining P_M this may not be strictly true and r_m may differ from zero by an amount probably not exceeding ±0·1 mm. For determining the relative change with wave-length of the value of p, this uncertainty is unimportant and we may put

$$p=\{(\log_{10}\eta)_{P_M}-(\log_{10}\eta)_Q\}/7\cdot56.$$

The mean values of p obtained in this way in three independent runs are shown in fig. 6. The general character of the variation of p with wave-length is similar to that obtained in the complete traverses (fig. 5). Owing to the method of taking readings, the relative values of p given in fig. 6 are more reliable than those of fig. 5, but if r_m is not in fact zero the absolute values of fig. 6 may be in error by a constant factor, probably lying within the range 1·08 and 0·92.

It may be suggested that p derived by the two methods need not be the same constant. It is easy to show, however, that if the luminous efficiency falls off in the same manner whatever the direction in which we move away from P_M, and if a formula of the type (3) holds good, then the curve of variation of $\log_{10}\eta$ on a line of any direction, and drawn through any point in the pupil, will yield the same value of p. Both the conditions mentioned are known to be approximately true.

It will be noted from Tables I and II that the photometric brightness B of the test field varied widely for the different wave-lengths. The variation of intensity expressed in energy units (U_λ) is less, but is still considerable. It is clear that if the magnitude of the effect varies with intensity the variation of p with wave-length shown in figs. 5 and 6 might be attributed wholly or in part to variations in the field brightness. A series of four runs was then made on the same lines as those reported in Table II, but keeping the photometric intensity of the matching field approximately constant. The mean data for these runs are given in Table III, and, in fig. 7, p is plotted against λ. The agreement between the values of figs. 6 and 7 is as good as can be expected with this kind of measurement, and we may conclude that the variation of p with wave-length is not to be explained as the result of variations of the field brightness.

Table II—Values of p deduced from the Relative Luminous Efficiencies for a Ray entering at P_M and a Ray entering 2·75 mm. to the Nasal Side of P_M

λ mμ	p (Mean data for three runs)	Conditions for one of the three runs*				
		λ mμ	$\Delta\lambda$ mμ	ρ	$\log_{10} U_\lambda$	$\log_{10} B$
410	0·0624†	420	12·9	0·25	$\bar{4}\cdot4$	$\bar{3}\cdot3$
427	0·0756	440	15·2	0·5	$\bar{4}\cdot7$	$\bar{2}\cdot4$
447	0·0731	460	10·9	1·0	$\bar{4}\cdot4$	$\bar{2}\cdot4$
467	0·0753	480	8·4	0·5	$\bar{4}\cdot8$	$\bar{1}\cdot2$
487	0·0695	500	7·4	0·5	$\bar{4}\cdot7$	$\bar{1}\cdot5$
507	0·0682	520	8·6	0·5	$\bar{4}\cdot8$	0·0
527	0·0646	540	9·8	0·5	$\bar{3}\cdot2$	0·5
547	0·0580	560	11·0	0·5	$\bar{3}\cdot3$	0·6
567	0·0566	580	12·5	0·5	$\bar{3}\cdot4$	0·6
587	0·0585	600	13·4	0·5	$\bar{3}\cdot5$	0·6
607	0·0577	620	15·0	0·5	$\bar{3}\cdot4$	0·3
627	0·0586	640	17·0	0·5	$\bar{3}\cdot5$	0·1
647	0·0625	660	18·8	0·5	$\bar{3}\cdot6$	$\bar{1}\cdot6$
667	0·0688	680	19·8	0·5	$\bar{3}\cdot7$	$\bar{1}\cdot2$
687	0·0684	700	22·0	0·5	$\bar{2}\cdot0$	$\bar{2}\cdot9$
707	0·0635	720	23·4	0·5	$\bar{2}\cdot0$	$\bar{2}\cdot3$
727	0·0631	740	23·3	0·5	$\bar{2}\cdot1$	$\bar{3}\cdot8$
747	0·0661					

* The conditions for the other two runs were not very different and need not be given.

† One observation only.

Table III—Values of p deduced from the Relative Luminous Efficiencies for a Ray entering at P_M and a Ray entering 2·75 mm. to the Nasal Side of P_M. Brightness B of Test Field approximately constant and equal to 0·1 c./ft.^{2}

λ mμ	$\Delta\lambda$ ($\rho=1$) mμ	p (mean data for four runs)
440	22·5	0·0799
480	33·4	0·0721
510	40	0·0659
550	40	0·0636
580	40	0·0588
610	40	0·0593
640	40	0·0629
680	40	0·0670
720	40	0·0625

Some additional runs were made, keeping the wave-length constant and varying the field intensity. For all wave-lengths, the effect as specified by p was found to be approximately independent of intensity over a considerable range. For wave-lengths in the middle of the spectrum there was an indication of a marked increase in the effect at very high brightnesses of the order of $B = 200$ c./sq. ft. This intensity could not be reached in the blue, blue-green and red. There was also some tendency for the effect to increase at very low intensities ($B = 0{\cdot}01$ c./sq. ft.) in the orange and red. As only a single run was made with each wave-length, the above observations are of a tentative character.

PART II—THE CHANGE IN COLOUR OF A MONOCHROMATIC LIGHT AS THE POINT OF ENTRY OF THE RAY IN THE PUPIL IS VARIED

Experimental

Attention was drawn in the Introduction to the change of colour which occurs when the traversing beam is moved across the pupil. For investigating this colour change, arrangements were made to enable the wave-length drum of spectrometer II to be operated by the subject who was then given the task of varying both the wave-length and intensity of the beam from spectrometer II until the two halves of the photometric field matched in brightness and colour. Owing to the use of Maxwellian view with beams of very small cross-section at the eye pupil, a perfectly uniform structureless field is not obtained even when both the images ω_1 and ω_2 are located at the centre of the eye pupil. As the traversing image ω_2 moves away from the centre the imperfections of the corresponding half field tend to increase, and at the extreme position of ω_2 the half field acquires a fuzzy appearance. The difficulties of making a colour and brightness match under these conditions are considerable. An additional difficulty is experienced in the blue and red ends of the spectrum owing to the rapid change of photometric intensity which occurs as the wave-length drum of the spectrometer is turned. This is a serious drawback to the method of colour matching used here, a method which was adopted only because it involved very little modification of the apparatus. Finally, in the green and blue-green the change of colour as the traversing beam is moved across the pupil is not a simple change of hue which can be eliminated by a change of wave-length, but is a change of both hue and saturation. Where this occurred, the subject adjusted wave-length and intensity to give the best hue and brightness match.

Results

If λ_2' is the mean setting of the wave-length of the traversing beam to give a colour and brightness match with the fixed beam, when the point of entry is at the centre of the pupil, and λ_2'' is the corresponding quantity when entry is at any point P of the pupil opening, then we shall express our results by giving the value of $(\lambda_2' - \lambda_2'')$. The results of a series of readings corresponding to two horizontal traverses through the pupil centre one in the direction nasal to temporal (circles), the other in the reverse direction (crosses), are shown in fig. 8. Each plotted point is the mean of four settings in which the difference between the maximum and minimum settings of λ_2'' equalled on the average 0·85mμ. The wave-length λ_1 of the fixed comparison beam entering through the centre of the pupil was 578·0mμ. With the traversing beam also entering centrally the mean setting equalled 578·8mμ. The difference is hardly more than the experimental error. In other cases somewhat larger differences were obtained, attributable to differences in the band width $\Delta\lambda$, slit ratio ρ and stray light glasses used in the two optical trains. Such differences need not occupy us here and we can confine our attention to the values of λ_2.

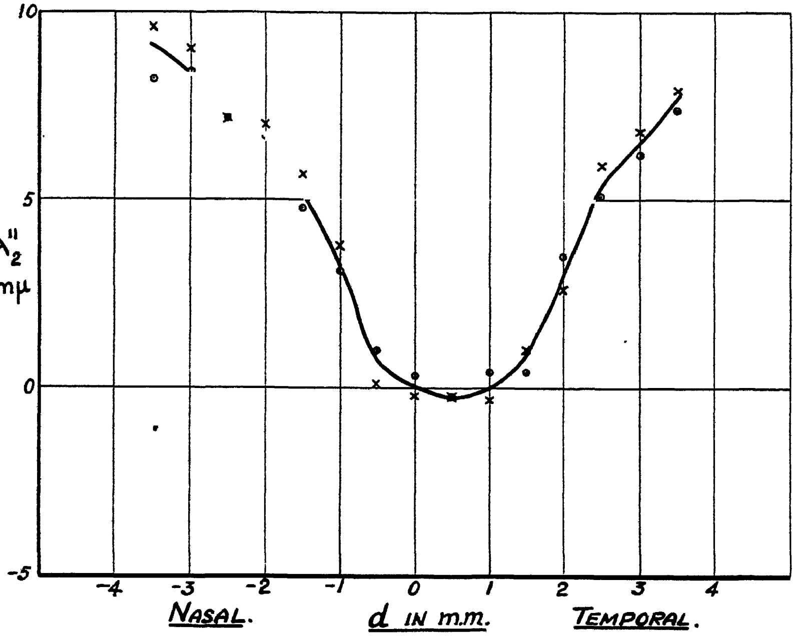

FIG. 8—Hue change with position of point of entry. $\lambda_2' = 578{\cdot}8\text{m}\mu$.

It will be noted that the curve of fig. 8 is approximately symmetrical about an ordinate through a point P_M at about 0·5 mm. to the temporal side of the pupil centre. It will be recalled that the curve of the intensity effect was found to be symmetrical about an ordinate similarly situated. The direction of the colour change is such that as the point of entry moves away from P_M the colour of the traversing beam appears to become relatively redder, so that its wave-length has to be reduced to maintain colour match with the comparison field. At this part of the spectrum a reasonably good colour match is obtainable wherever the traversing beam enters the pupil. Thus the colour change is in the main a hue change. The change at its greatest equals about 9 mμ. As the smallest perceptible hue difference at $\lambda = 579$ mμ is of the order of 1·5 mμ, it is clear that the effect at this wave-length is not difficult to observe.

The results for a series of horizontal traverses at wave-length ranging from 656 to 457 mμ are given in Table IV and fig. 9. In the table a comment is given for each wave-length on the character of the colour match obtainable as the traversing spot moves away from the pupil centre. At $\lambda_2' = 500$ mμ the greater saturation of the traversing beam when it enters near the edge of the pupil is well marked and a similar effect is in evidence for $\lambda_2' = 478$ mμ. With the matches described as fairly satisfactory it was difficult to state precisely in what manner the match fell short of perfection.

In fig. 9 the origin of the ordinate axis for each curve is to be taken as the point where the curve intersects the ordinate through $d = 0$. An examination of the figure reveals that most of the curves are very approximately symmetrical about an ordinate through a point displaced to the temporal side of the pupil centre by about $\frac{1}{2}$ mm. For the curves for $\lambda_2' = 636$ and 656 mμ, however, a pronounced asymmetry is present. It will be noted that although the curves for $\lambda_2' = 657$ to $\lambda_2' = 562$ mμ and for $\lambda_2' = 478$ and 457 mμ show a fairly steady increase in $(\lambda_2' - \lambda_2'')$, starting from the value of d at which $(\lambda_2' - \lambda_2'')$ is a minimum and moving away either to the temporal or nasal side, the curves for $\lambda_2' = 542$, 522 and 500 mμ are of a more complex character. The manner in which the magnitude of the hue change varies with wave-length is best seen with the aid of fig. 10 in which the values of $(\lambda_2' - \lambda_2'')$ are plotted against λ_2' for $d = -3·5$ mm. (nasal), $d = -2·5$ mm. (nasal), $d = +3·5$ mm. (temporal). Owing to the displacement of the centres of symmetry of the curves of fig. 9 to the temporal side, the values for $d = -3·5$ mm. correspond to maximal effect. The values for $d = -2·5$ mm. and $+3·5$ mm. would be the same if all the curves were symmetrical about the ordinate $d = +0·5$ mm. Examining the graphs of fig. 10 it is clear that for these d values the hue change has a maximum in the neighbourhood of

TABLE IV—VALUES OF $(\lambda_2'-\lambda_2'')$ IN Mμ. HORIZONTAL TRAVERSE THROUGH THE PUPIL CENTRE. SUBJECT W. S. S. LEFT EYE. PUPIL DILATED WITH EUPHTHALMINE

λ_2' (mμ)	656	636	622	601	579	562	542	522	500	478	457
$\Delta\lambda_2\,(\rho=1)$	10·0	10·4	10·0	10·0	10·0	10·0	10·0	10·2	9·8	10·0	10·1
$\log_{10} U_\lambda$	$\bar{3}{\cdot}1$	$\bar{3}{\cdot}0$	$\bar{4}{\cdot}9$	$\bar{4}{\cdot}9$	$\bar{4}{\cdot}7$	$\bar{4}{\cdot}9$	$\bar{4}{\cdot}3$	$\bar{4}{\cdot}5$	$\bar{4}{\cdot}1$	$\bar{4}{\cdot}1$	$\bar{4}{\cdot}3$
$\log_{10} B$	$\bar{1}{\cdot}2$	$\bar{1}{\cdot}6$	$\bar{1}{\cdot}8$	0·0	0·0	0·2	$\bar{1}{\cdot}6$	$\bar{1}{\cdot}6$	$\bar{2}{\cdot}9$	$\bar{2}{\cdot}5$	$\bar{2}{\cdot}4$
d (mm.)											
−3·5 nasal	12·6	6·6	8·3	8·8	8·9	7·1	3·1	—	2·9	6·2	4·6
−3·0 ,,	8·7	6·4	6·5	7·5	8·7	6·9	1·8	−2·2	2·3	6·1	6·2
−2·5 ,,	11·1	6·1	7·1	6·8	7·2	5·6	2·5	−0·4	−0·6	6·3	4·7
−2·0 ,,	9·1	5·2	4·5	5·4	6·8	5·7	2·3	0·6	+0·1	4·8	4·8
−1·5 ,,	8·2	2·2	2·7	4·1	5·2	4·1	2·8	1·7	0	3·1	2·8
−1·0 ,,	3·3	0·4	1·1	1·5	3·4	2·5	3·1	2·0	0·5	1·1	0·7
−0·5 ,,	3·1	0·8	0·7	0·8	0·6	1·3	1·2	0·4	0·9	0·9	−0·5
0 ,,	−0·3	0·4	0·3	−0·4	0	0·1	−0·1	0·2	−0·1	0·1	0·2
0·5 temporal	−0·5	−0·5	0	−0·2	−0·2	−0·9	0·1	−0·5	−0·7	−0·4	0·7
1·0 ,,	−1·3	0·6	−0·7	−0·2	0	0	−0·7	1·0	−0·1	−0·8	0·5
1·5 ,,	−2·3	−1·1	1·0	0·2	0·7	1·1	0·9	0·6	−0·2	−0·3	1·3
2·0 ,,	−0·3	−1·8	0·5	1·0	3·0	3·8	1·8	0·6	−0·4	0·9	2·3
2·5 ,,	1·3	−0·6	2·8	3·4	5·5	4·2	2·0	1·1	−0·5	1·9	3·4
3·0 ,,	2·7	2·0	4·1	5·4	6·5	4·7	2·4	0·6	0·6	2·9	5·1
3·5 ,,	3·9	2·2	5·4	6·8	7·6	5·6*	2·6	−0·7	2·4	4·4	5·8
Remarks on colour match	Satisfactory	Satisfactory	Satisfactory	Fairly satisfactory	Fairly satisfactory	Fairly satisfactory	Fairly satisfactory	Fairly satisfactory	ω_2 more saturated than ω_1	ω_2 more saturated than ω_1	Satisfactory

* $d=3{\cdot}25$.

$\lambda_2' = 590\,\mathrm{m}\mu$, a minimum at $\lambda_2' = 520\,\mathrm{m}\mu$, where the hue change is negative, i.e. the traversing beam becomes relatively bluer, and, probably, a further maximum in the neighbourhood of $\lambda_2' = 470\,\mathrm{m}\mu$. Little weight can be attached to the high values of the points for $\lambda_2' = 657\,\mathrm{m}\mu$, as the difficulties of measurement are here particularly severe, largely due to the falling off of hue sensitivity in the red end of the spectrum.

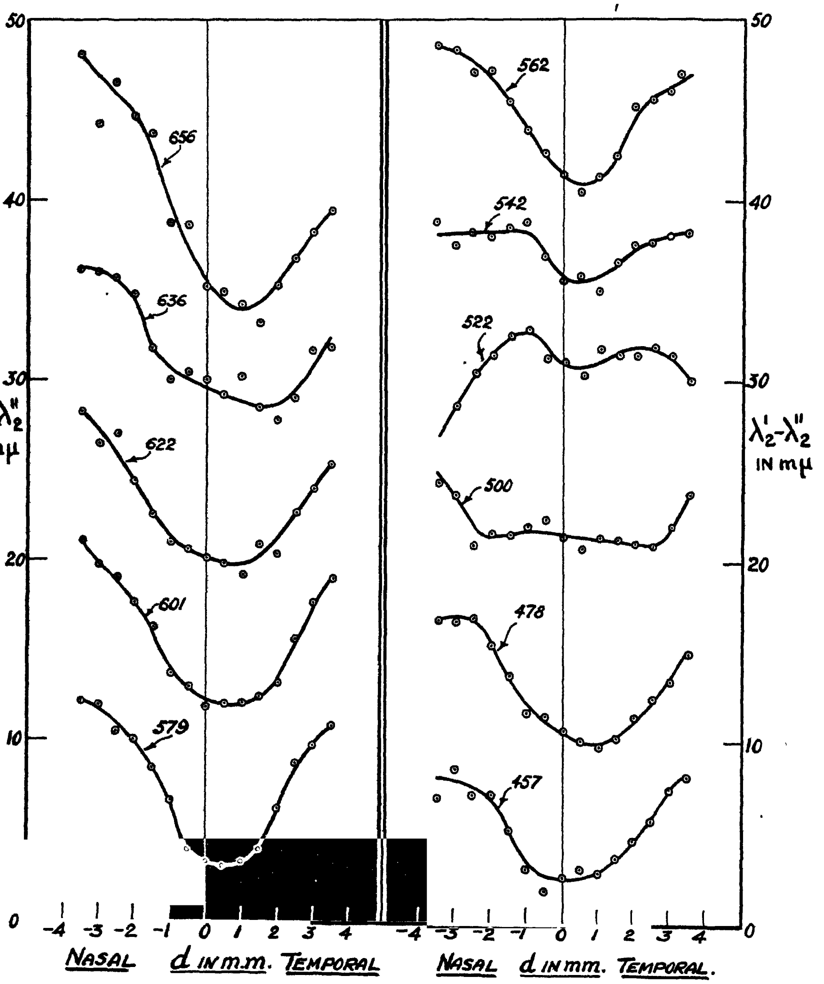

FIG. 9

As a consequence of the complexity of shape of some of the curves of fig. 9, the graph connecting $(\lambda_2'-\lambda_2'')$ with λ_2' at a particular d value is not in general obtainable by a proportional change in the ordinates of the corresponding graph for any other d value. This is clearly shown by a comparison of fig. 10 with fig. 11 in which $(\lambda_2' - \lambda_2'')$ is plotted against λ_2' for $d = -1$ mm. (nasal) and $+2$ mm. (temporal). The difference in shape of the graphs of figs. 10 and 11 in the region $\lambda_2' = 500$ to $550\,\text{m}\mu$ is well marked.

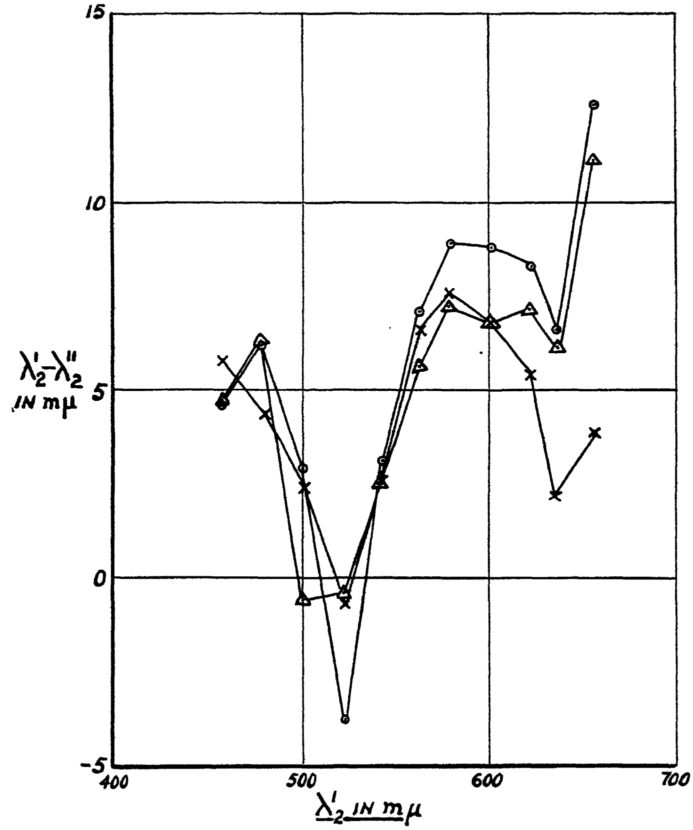

FIG. 10—⊙ $d = -3{\cdot}5$ mm. nasal; △ $d = -2{\cdot}5$ mm. nasal; × $d = +3{\cdot}5$ mm. temporal.

All the above results refer to one eye. Qualitative observations on other eyes confirm the existence of a colour change as the point of entry is varied and for one of these eyes (B. H. C. right) the graph of fig. 12 represents a single series of determinations of $\lambda_2' = \lambda_2''$ for a point at $d = -2{\cdot}5$ mm. Compared with the graphs of fig. 10, we note the very large negative value at $\lambda_2' = 520\,\text{m}\mu$ and the larger positive values in the blue and blue-green than in the yellow. There is, however, a generic similarity. It is hoped to continue the investigation of the colour change effect using a trichromatic colorimeter, and the behaviour of a number of eyes will then be examined.

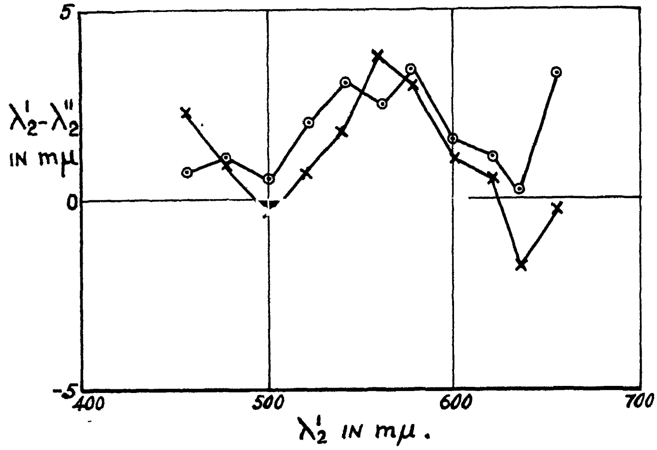

Fig. 11—⊙ $d = -1$ mm. nasal; × $d = +2$ mm. temporal.

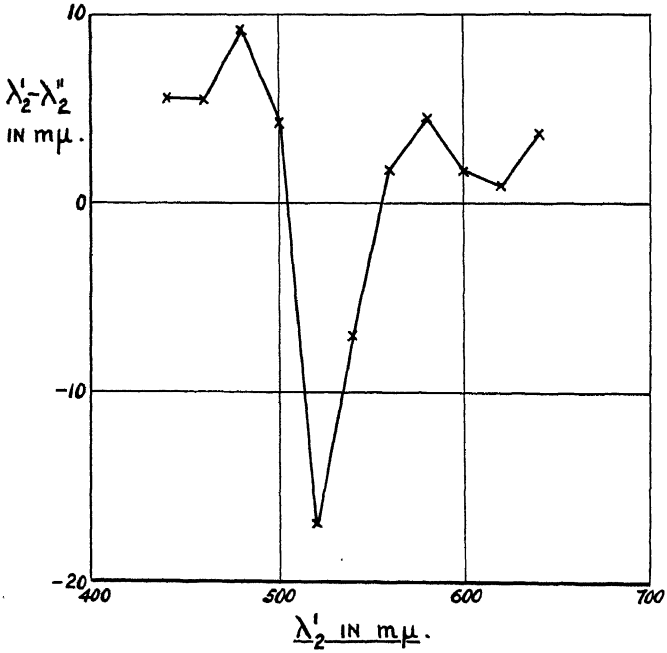

Fig. 12—× $d = -2{\cdot}5$ mm.

Colour Changes arising as Artifacts of the Method

It has been taken for granted up to now that the observed colour change is due to the fact that the colour of a physically homogeneous radiation incident on a given area of the retina depends on its point of entry in the eye. Actually spectrum bands of a total width equal to about 10 mμ were used in the measurements and there are three ways in which a colour change might conceivably be produced other than by an effect of the kind just described. The three possibilities are the following:

(1) If the different wave-lengths in the traversing beam are reduced in apparent intensity to different extents when the image ω_2 moves from the centre to the edge of the pupil, the resultant colour of the field will change. This change will be due to the variation with wave-length of the pupil intensity effect which is seen from fig. 6 to be greatest in the neighbourhood of $\lambda = 640$ mμ and $\lambda = 515$ mμ. Calculation shows that at these wave-lengths under the conditions of the experiments, the resulting hue change is at most of the order of 0·1 mμ, which is negligible.

(2) Selectivity of the wedge W_3 may produce a colour change in precisely the same way as selectivity in the pupil intensity effect. Calculation shows, however, that the resultant hue changes will be even smaller than in the latter case.

(3) The image ω_2 is in fact a small section of the spectrum produced by spectrometer II, and the wave-length in the image varies (in our case it increases), in passing from the left-hand to the right-hand edge, by the amount $\Delta\lambda/2$. When ω_2 is displaced to the temporal side of the left eye, the left-hand edge will clearly be subject to greater pupil intensity effect than the right-hand edge, because it enters the pupil at a more peripheral point. Thus, there will occur a change in the colorimetric hue of the field, each part of which is illuminated by the whole of the image ω_2. In a traverse of the pupil this change will be of opposite sign on either side of the point at which the pupil intensity effect is maximal. The magnitude of this effect can also be calculated and it is found that under the conditions used, the resultant hue change is of the order of 0·2 mμ at most.

Thus, none of the above colour changes arising as artifacts of the method is nearly large enough to explain the observed effects. The changes are in fact too small to warrant the application of any corrections to the data.

By substituting for the continuous light source S a sodium or mercury vapour lamp and isolating individual lines in the spectrum, the change of colour with point of entry was obtained with light of a very high degree of physical homogeneity. The effect is easiest to observe with the sodium D lines and with the mercury line at 491·6 mμ.

DISCUSSION OF RESULTS

It is highly probable that the change in luminous efficiency of a monochromatic ray with change of the point of entry in the pupil (intensity effect) and the change in colour of the ray (colour effect) are closely related in their mode of origin. That such is the case is strongly suggested by the fact that most of the curves of $(\lambda_2' - \lambda_2'')$ against λ_2' show a displacement of their axes of symmetry in the same direction and of about the same amount as shown by the curves of the intensity effect. The explanation of the colour effect is certainly to be sought in the retinal mechanism, for it is clear that an increased absorption in the optic media of the eye can only reduce the intensity of a monochromatic ray, and when this is compensated for by increasing the intensity of the incident light the colour impression must be the same. It follows that in all probability the intensity effect is also retinal. This conclusion is clinched by measurements due to Crawford of the intensity effect in determinations of foveal and extra-foveal threshold values.*

The hypothesis has been advanced (Stiles and Crawford 1933, p. 446) that the intensity effect is due to the ensheathing of the cones by pigment contained in processes of the pigment epithelium intruded between the cones. Wright and Nelson (1936) suggest on the other hand that a difference in the refractive index of the cone and of its surrounding medium may provide the explanation of the intensity effect. In the following discussion where a picture of the mechanism is required the pigment sheath hypothesis is employed. The experimental evidence is however insufficient to decide between the two views mentioned and further work may reveal other possibilities.

Both the variation of the intensity effect with wave-length and the colour effect can be represented formally in terms of the trichromatic theory in the following manner. Suppose light of wave-length λ, incident through the centre of the pupil, has the trichromatic co-ordinates r_λ, g_λ, b_λ in a particular system of primaries (unitary stimuli) R, G, B for which the luminosity coefficients equal L_r, L_g and L_b respectively. We may suppose that when the point of entry of the incident light is changed, keeping its intensity constant, the response of the eye changes in a manner corresponding to the reduction of stimulation by the R, G, B primaries to fractions x_λ, y_λ, z_λ respectively of their previous values. The intensity effect specified by η, the ratio of the brightness when the light enters in the displaced position to the brightness for central entry, will equal

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* These experiments will be described in another paper.

$$\eta = \frac{x_\lambda r_\lambda L_r + y_\lambda g_\lambda L_g + z_\lambda b_\lambda L_b}{r_\lambda L_r + g_\lambda L_g + b_\lambda L_b}. \qquad \text{......(4)}$$

The colour change will correspond to a shift in the colour triangle, from the point r_λ, g_λ, b_λ to the point r'_λ, g'_λ, b'_λ, where

$$r'_\lambda = \frac{x_\lambda r_\lambda}{\Sigma}, \quad g'_\lambda = \frac{y_\lambda g_\lambda}{\Sigma}, \quad b'_\lambda = \frac{z_\lambda b_\lambda}{\Sigma},$$

and

$$\Sigma = x_\lambda r_\lambda + y_\lambda g_\lambda + z_\lambda b_\lambda.$$

For the observed intensity effect and colour effect which occur for a particular wave-length and for a particular point of entry in the pupil, the corresponding values of x_λ, y_λ, z_λ can always be determined. That this can be done involves no hypothesis about the visual mechanism of the retina other than that implied by the possibility of three colour matching. For every different set of primaries which may be chosen, x_λ, y_λ and z_λ will have different values.

According to the trichromatic theory, there are three types of cone concerned in colour vision. As a first step towards the explanation of the colour effect and the variation of intensity effect with wave-length, the simplest assumption is that the cones of the three types would give different intensity effects if it were possible to stimulate one type independently of the others. In terms of the pigment sheath hypothesis this might mean that the cones of one type were longer and thinner or more deeply embedded in the pigment sheathing than cones of another type. It would then follow that if the primaries *R*, *G*, *B* had been so chosen that each stimulated one and one only of the cone types, the coefficients x_λ, y_λ, z_λ would correspond respectively to the reduction in response of the three types of cone. The possibility that this reduction might be independent of wave-length for all three types was first investigated, i.e. x_λ, y_λ, z_λ were put equal to constants x, y and z respectively.

Adopting the cone response curves derived by Wright* (1934) from his colour adaptation experiments, the ratios y/x and z/x were chosen to give correctly the observed hue changes at $\lambda = 486\,\text{m}\mu$ and $\lambda = 590\,\text{m}\mu$ shown in fig. 10 for $d = -3{\cdot}5$ nasal. The values necessary were $y/x = 0{\cdot}773$ and $z/x = 0{\cdot}380$. Using these values, the hue changes for other wave-lengths in the spectrum were derived. The curve of hue change obtained in this way and the curve of observed hue change for $d = -3{\cdot}5$ nasal are shown in fig. 13. The curves show complete lack of agreement. Moreover, the calculations indicate that for wave-lengths below 550 mμ the ray entering at the displaced position should be a little less saturated than the spectrum colour which it matches in hue. The opposite was found to be the case experimentally. Finally, the relative change in η was determined from equation (4). Fig. 14 gives curve of $\log_{10}\eta$ against λ for $d = -3{\cdot}5$ mm. (nasal) obtained from the experimental values of p shown in fig. 6, together with the theoretical curve from equation (4) fitted at $\lambda = 530$ mμ by putting $x = 0{\cdot}095$. The two curves show little agreement.

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* The fundamental response curves are depicted in figs. 8 and 9 from which the values here used have been obtained.

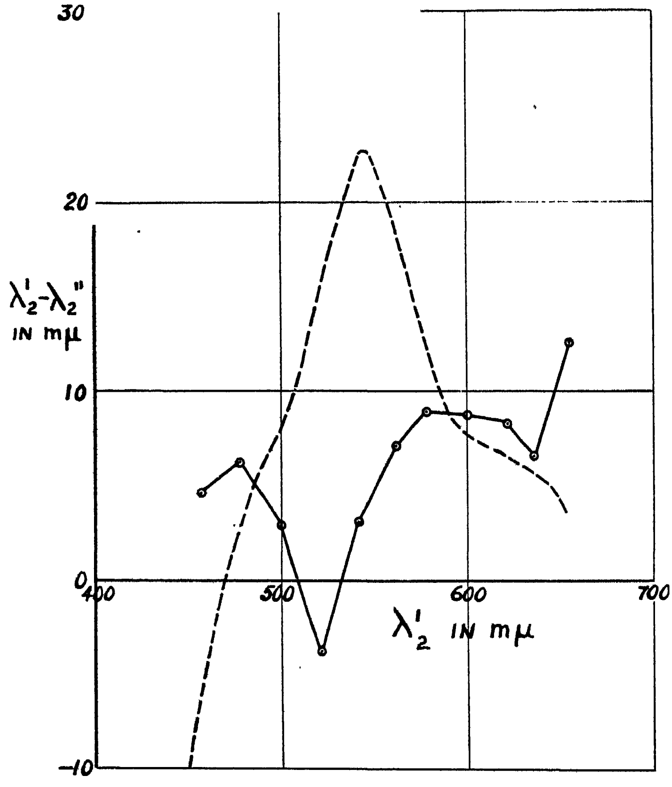

FIG. 13——----- curve computed from Wright's fundamental response curves, x, y and z assumed constant; ⊙ observed hue change for $d = -3{\cdot}5$ mm. (nasal).

Similar calculations were tried using Hecht's response curves for the cone primaries. These also failed to yield the experimental curves of the colour and intensity effects. It would be possible to choose a set of primaries to give the agreement sought, but such a derivation is not warranted (*a*) because of the scanty data on the colour effect available at present, (*b*) because it appears from a preliminary exploration that the primaries so obtained would differ radically from those acceptable on other grounds.

Abandoning the view that x, y and z are constants, we might assume them all to be proportional to a single function of wave-length. This would enable the variation of $\log_{10}\eta$ with λ to be adjusted to agree with the experimental

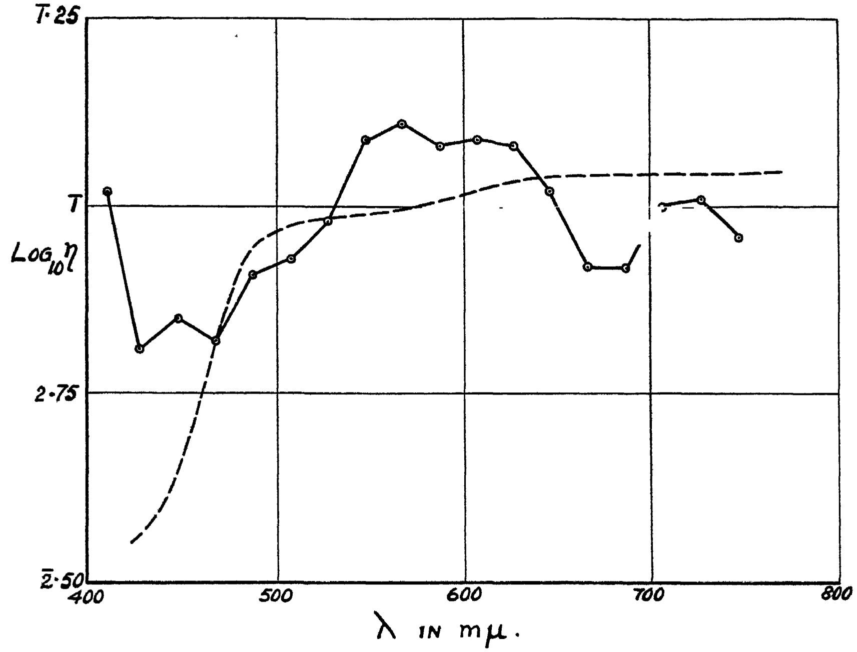

FIG. 14 – – – – – – curve computed from Wright's fundamental response curves, x, y and z assumed constant; ⊙ values based on experiment.

curve, but the difficulty of the hue change would remain. It appears most probable therefore that the relative reductions in response of the different types of cone for light incident obliquely must vary in passing through the spectrum, i.e. y/x and z/x must be functions of wave-length. A physical picture of how this might come about is obtained in the following way. Suppose each cone to be filled with a photochemical substance which attenuates the light in its passage through the cone. As a consequence the photochemical action will also decrease in amount in passing from the inner to the outer extremity of the cone. Since the pigment sheath gives rise to the intensity effect by shielding the outer extremity of the cone from obliquely incident light it follows that the intensity effect will be less, the less the contribution of the outer extremity of the cone to the photochemical action. Thus at the wave-length at which the absorption of the photochemical substance is greatest the intensity effect will be smallest and there will be a variation of intensity effect with wave-length corresponding to the absorption curve of the photochemical substance. Admitting that the different types of cone must contain photochemical substances of different spectral absorption curves it is clear that the ratio of the intensity effects for the different types of cone will vary with wave-lengths. This means simply that y/x and z/x will vary with wave-length.

This picture is of course only one of several that might be developed. The measurements of the colour effect which it is hoped to carry out with a trichromatic colorimeter may be expected to throw further light on the matter.

The writer has much pleasure in acknowledging the assistance rendered by Mr. G. W. Gordon-Smith in carrying out the measurements described.

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The Linkage between the Genes for Colour-blindness and Haemophilia in Man

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It is well established that colour-blindness and haemophilia are due to sex-linked genes. These genes appear to manifest themselves in all males who carry them. In women the gene for haemophilia is probably always recessive, the cases of alleged haemophilia in heterozygous women being very doubtful. On the other hand, colour-blind women whose putative fathers are not colour-blind occur too frequently to be explained by illegitimacy (Bell 1926). So colour-blindness is probably not always recessive. On the other hand, women homozygous for the gene appear always to be colour-blind. No cases of incomplete recessiveness occur in the new pedigrees here presented. It will be assumed that a woman who is not colour-blind is not homozygous for the gene for colour-blindness.

There are two distinct forms of colour-blindness, namely protanopia ("red-blindness") and deuteranopia ("green-blindness"). According to Waaler (1927) the genes determining them form a series of five allelomorphs with the normal gene, and those for protanomalia and deuteranomalia. Haldane (1935) suggested that there are at least two different allelomorphic genes for haemophilia.

Morgan (1910) showed that in *Drosophila melanogaster* genes which are sex linked, and therefore completely linked in spermatogenesis, are partially linked in oogenesis. This principle has since been extended to other species of *Drosophila*, and *mutatis mutandis* to pigeons (Cole and Kelley 1919), to poultry and other organisms; and on the basis of the data so obtained the chromosomes have been mapped.

It is important to demonstrate that the principles of linkage which have been worked out for other animals also hold good for man. The choice of colour-blindness for one of the two genes whose linkage was to be investigated is obvious. Over 2 % of human males are colour-blind, and a distinctly larger fraction are anomalous trichromats. Of the remaining sex-linked abnormalities some are very rare, whilst others affect the eyes and would thus lead to confusion, or at best difficulty, in the detection of colour-blindness. Haemophilia is not excessively uncommon, and could have no effect on the detection of colour-blindness. Moreover, certain physicians specialize in its treatment, and thus considerable numbers of cases are available. It was decided to seek for haemophilics, to test their colour vision and that of their non-haemophilic brothers, and to follow up the family history if both conditions were found in the same family.

Addresses of haemophilics were kindly sent to us by a number of physicians, including Dr. Macfarlane of St Bartholomew's Hospital, Dr. Cockayne of Great Ormond Street Hospital, Dr. Miller of St Mary's Hospital, and Professor Bulloch of the London Hospital. Dr. W. D. Wright of the Royal College of Science, South Kensington, undertook to test the colour vision of these patients and their brothers for us.

The cases which emerged from this preliminary enquiry were seventeen haemophilics and thirteen unaffected brothers of haemophilics. Among these Dr. Wright found deuteranopia in two haemophilics of different families. The other members of these families were then followed up as far as possible, and examinations of colour vision were made on all available members who were liable to inherit the defect, and occasionally on others. For this purpose Ishihara's tests were used. In no case was the diagnosis in doubt. We have to acknowledge with gratitude a grant from the Medical Research Council for the expenses involved in this work.

We owe two other pedigrees to the courtesy of colleagues, Dr. W. J. B. Riddell of Glasgow and Dr. C. L. Birch of Chicago. In each case the investigation is still incomplete and full accounts will be published elsewhere. They have, however, permitted us to publish the parts of their pedigrees which are relevant to our enquiry. Finally, Dr. Madlener of Kempten (Germany) most kindly gave us further information concerning members of the family previously published by him (Madlener 1928) in which the first evidence of linkage between the two genes was obtained. Thus with the pedigree published by Davenport (1930) we have information concerning six families in which these two conditions occur. The information is sufficient to leave no doubt as to the existence of linkage, but its intensity is not yet established with accuracy.

DESCRIPTION OF NEW PEDIGREES

The pedigree A, fig. 1, including the colour-blind haemophilic IV 9, had already been investigated with regard to haemophilia, and was given to us by IV 9, a patient of Dr. Macfarlane's discovered by Dr. Wright to be deuteranopic. We examined the colour vision in eight more members of this family, and found the pedigree, so far as we were able to verify it, correct in all particulars amongst the descendants of II 1 and II 2. The haemophilics are limited to one branch of the family, namely the descendants of III 2.

IV 9 is an intelligent and co-operative man, and personally arranged for the examination of his cousins and nephews. Unfortunately a family quarrel prevented our testing the vision of the non-haemophilic brother, IV 2, who wrote that "though I bear at present the name of *X*... I consider myself of no blood relationship to that family and no useful purpose would be served by visiting me". We called in order to try if persuasive methods could secure a valuable addition to our knowledge, but were not admitted to his house. III 2 has two living brothers who are not haemophilic, but we were unable to examine their colour vision.

The most interesting point in this pedigree is the limitation of the haemophilia to one branch of the family. The two normal nephews of IV 9, who had a chance of being haemophilic and colour-blind, were actually neither. Thus no crossing-over had occurred in the family, the absence of haemophilia in the cousins of IV 9 being explicable by mutation. The information regarding linkage is, however, meagre.

The pedigree B, fig. 2, provides a much better illustration of linkage.

V 1 and V 2 are cases of haemophilia under the care of Dr. Macfarlane. They appear to be typical examples of the disease, with a history of repeated haemorrhages into their joints; V 2 has recently been away from work for more than 11 weeks following a slight injury; the bleeding was so severe as to soak through his mattress and drip on to the floor.

Starting from these men, who combined haemophilia and deuteranopia, the family was followed, and seven further males who, from their relationship to V 1, were potentially haemophilic or colour-blind, were examined. The family is somewhat scattered, one member living in Devonshire.

V 3 died in early infancy having shown no symptoms of haemophilia.

V 6 died in infancy from haemorrhage following circumcision. The parents were told at hospital that, had the family history been known, this operation would not have been performed. It is believed, probably correctly, by the family that this child had haemophilia.

III 1 is said to have been a very severe case of haemophilia. One of his sisters reports that he had a stiff leg from the age of 20, the result of repeated haemorrhages into the joints. He died at about 32. III 1 was the only haemophilic child of his mother. Her six sons by a different father were all healthy. We were able to see three of these and the children of two of them. III 5 is aged about 78. The remaining three brothers, III 2, are dead or inaccessible, but have shown no signs of haemophilia. Information concerning this branch of the family was given by V 1 and V 2. They are intelligent young men, ready to co-operate; they took very considerable trouble to seek out their relations, many of whom they had never seen before, and to arrange for us to see them and test their colour vision. These brothers also put us in touch with III 18 of another branch of the family.

III 18, aged 60, is a frail-looking intelligent little shoemaker. He was married late and had lived at home with his mother and his three affected brothers for a long period of years, when he says the endless topic of conversation was the presence and source of haemophilia in the family. He volunteered the information that members of the family had the disease either severely or not at all, and said there could not be the slightest doubt with regard to who was, or was not, affected in his family. He described the disease in his brothers and uncle. According to him his mother had had four or more brothers, some of whom were certainly haemophilic; perhaps all had the disease, but he cannot be quite sure of this; he also can only be sure with regard to one affected brother of his maternal grandmother. He says that they have never been able to trace the transmission of the disease through a male in this family, except in the case of III 1. He knew of the affection of III 1, but only heard through our visit of the affection of his grandsons, V 1, V 2 and V 6.

III 18 was a little uncertain with regard to the children of II 6; he was assured that all members of her family, except a daughter, had died in infancy; he believed that all her sons were haemophilic. He had lost touch with the family of his sister, III 14, who died many years ago, but fortunately a letter to the old address brought an immediate response from IV 20, who was still living there.

IV 20 gave an account of his sibship agreeing in each particular with his uncle's, thus showing the reliability of the latter's statements. He brought with him a medical certificate affirming his haemophilia, and has evidently suffered severely from the disease.

We have thus found that all the four living haemophilics in this pedigree are colour-blind. On the other hand, four non-haemophilic brothers and one non-haemophilic son of a haemophilic's sister are not colour-blind. Thus in this family the linkage between haemophilia and colour-blindness has been complete.

The pedigree described in fig. 3, which we owe to Dr. Riddell, contains an example, so far unique, of two brothers, both of whom are colour-blind but only one haemophilic. The haemophilia in this family is confined to one sibship whose mother, II 4, has three brothers and seven sister's sons, none of whom has shown symptoms of haemophilia. Their colour vision has not yet been investigated.

With regard to the pedigree of fig. 4, Dr. Birch states that there is a tradition in the family concerned that individuals with haemophilia have normal colour vision, while males who are not haemophilic are colour-blind. She has only been able so far to test the validity of this tradition in two cases. Her patient III 1, a haemophilic, was found to have normal colour vision, while III 3, one of his non-haemophilic brothers, was found to be colour-blind, apparently deuteranopic. II 4 and III 4 are reported by their relatives to be colour-blind, while III 2 is believed to have had normal colour vision. Dr. Birch hopes to test the colour vision of II 3, II 4, II 5, III 4 and IV 4, and to publish the full results in a forthcoming memoir on the subject of haemophilia.

MUTATION

Haldane (1935) pointed out that haemophilia would be rapidly extinguished by natural selection if the gene did not constantly arise anew by mutation. He estimated the frequency of this event in the population of London at about 2×10^{-5} per X-chromosome per generation. A communication by Dr. Birch as to its frequency in Illinois suggests a distinctly higher value, of the order of 10^{-4}, for that population.

The pedigrees of figs. 1 and 3 are most readily interpreted on the hypothesis of mutation. III 2 of fig. 1 has borne three haemophilics and one normal son; she is therefore either heterozyous $\left(\frac{+}{\mathbf{h}}\right)$ or a mixture of homozygous and heterozygous tissue $\left(\frac{+}{+} \text{ and } \frac{+}{\mathbf{h}}\right)$.

Now suppose that the gene **h** present in the haemophilic descendants of III 2 did not originate by mutation in any member of the pedigree shown, it follows that I 2, II 2, and III 2 were heterozygous for it, since I 1 and II 1 were normal. In view of the accuracy of the statements of III 2 and IV 9 where they could be verified, and their openness in discussing the problem, we think that their statements as to the absence of haemophilia in this part of the pedigree may be accepted.

We can now ask, what is the probability that two heterozygous women, I 2 and II 2, should have had no haemophilic sons, and that their daughters, other than III 2, should have been equally fortunate? It must be stated that II 3 represents three sons, all of whom survived long enough to marry. Of the two daughters, II 4, one had two sons surviving infancy, and a daughter with two sons; the other had one son, and two daughters with one and three sons respectively. We shall neglect III 3 who died in infancy.

First consider the probability apart from the information supplied by linkage. The probability that the two mothers should have had five normal sons is 2^{-5}. The probability that the daughter of a heterozygous woman should have n normal sons is $\frac{2^n+1}{2^{n+1}}$. The values of n for the seven daughters, II 2, III 2, III 5, III 7, III 8, III 11 and III 13 are 2, 1, 1, 4, 1, 1 and 3. The probability is therefore $3^6 \times 5 \times 17 \times 2^{-25}$, or 0·00185. This would be slightly lessened if we considered the further information provided by daughter's daughters' sons.

It can be considerably diminished in view of the evidence as to the linkage between the genes **c** and **h**, presented later. In the sons of III 7, III 8 and III 13 colour-blindness appears without haemophilia, while in IV 9 both are combined. Hence in the oogenesis of either II 2 or III 2 the genes must have crossed over. The probability of this event is about $2x$, where x is the small frequency of crossing-over. Taking x as 0·05, the probability is reduced to 0·0002 approximately.

In Riddell's pedigree, fig. 3, either mutation occurred, or I 2 was heterozygous. As she had three normal sons and her two daughters, II 6 and II 7, had four and three sons respectively, the probability of this is $9 \times 17 \times 2^{-12}$, or 0·037. So here the evidence for mutation is strong, but by no means conclusive.

In Green's case, Davenport (1930) and Haldane (1935) have pointed out that there is very strong evidence for mutation, based on relatives not shown in fig. 6. The probability is $5 \times 2^{-10}x$, or about 0·00025.

Thus out of six pedigrees chosen because colour-blindness occurred in them, three show evidence of mutation varying from strong to almost conclusive. This is a much higher proportion than occurs in the bulk of the published material. The reason is, we think, obvious. Accounts of sporadic cases are less likely to be published than of families containing many haemophilics. In the investigation of haemophilia the stress has so far been laid on its heredity rather than on its origin.

TERMINOLOGY

The genes for colour-blindness and haemophilia will be represented by the letters **c** and **h**. The fact that the latter probably and the former certainly has several allelomorphs is irrelevant. Their normal allelomorphs will be represented, using Morgan's symbolism, by +. Thus a man is either

$++$ (normal), $\mathbf{c}+$ (colour-blind), $+\mathbf{h}$ (haemophilic), or $\mathbf{ch}$ (colour-blind haemophilic). A woman, being diploid for these genes, is $\frac{++}{++}$, $\frac{++}{\mathbf{c}+}$, $\frac{++}{+\mathbf{h}}$, $\frac{++}{\mathbf{c}\,\mathbf{h}}$ or $\frac{+\mathbf{h}}{\mathbf{c}+}$, if phaenotypically normal. The latter two genotypes will differ in their progeny. The sons of $\frac{++}{\mathbf{c}\,\mathbf{h}}$ women will be mainly normal or colour-blind haemophilic. Those of $\frac{+\mathbf{h}}{\mathbf{c}+}$ women will be mainly haemophilic or colour-blind, but rarely both or neither. The other possible female genotypes are $\frac{\mathbf{c}+}{\mathbf{c}+}$, $\frac{\mathbf{c}+}{\mathbf{ch}}$, $\frac{+\mathbf{h}}{+\mathbf{h}}$, $\frac{+\mathbf{h}}{\mathbf{c}\,\mathbf{h}}$ and $\frac{\mathbf{ch}}{\mathbf{ch}}$. It is possible that the $\frac{\mathbf{h}}{\mathbf{h}}$ genotypes are inviable. In any case they are very rare indeed.

Besides these types others may possibly exist. The gene for haemophilia appears to arise anew by mutation. If the mutation only occurs at meiosis, the above list is complete. If, however, it arises at a somatic division, mosaics may exist. Such mosaics, if male, would probably not be haemophilic. If haemophilia is due to a lack of some substance in the plasma, this would be supplied by the non-haemophilic tissues of a mosaic man. Such a man might transmit the gene to all, some, or none of his daughters according as all, some, or none of his testicular tissue carried the gene. A mosaic woman would not be haemophilic, but might transmit haemophilia to less than half of her sons. It is possible that III 2 in fig. 1 and II 4 in fig. 3 were of this type.

THE DETECTION AND ESTIMATION OF LINKAGE

Let x be the frequency of recombination between the loci of **c** and **h** so that the cross-over percentage is $100x$. Let $y = 1-x$.

It is our task to obtain as much information as possible from the data about the frequency x of crossing-over between the loci of the genes **c** and **h**. We have on the one hand to find out whether its estimated value departs significantly from $\frac{1}{2}$, its value in the absence of linkage, and on the other to obtain the best estimate of its true value. Our task is complicated by four considerations:

1. The gene **h** may arise by mutation. While this is a rare event in the population as a whole, it is frequent among the immediate ancestors of haemophilics, since, as has been shown by Haldane (1935), the frequency of mutation is about one-quarter of the frequency of the disease.
2. The gene **c** is not very rare in the population. It is excessively unlikely,

in the absence of close inbreeding, that haemophilia should arise from two different sources in one pedigree. Protanopia or deuteranopia might do so with a probability which is by no means negligible.

3. The estimation of x by any of the classical inverse probability methods involves the presupposition that all values of x in the neighbourhood of that found are equally probably *a priori*. We shall see that this is not so.

4. The sample observed is still so small that the probability distribution of x about its estimated value is far from normal. We cannot, therefore, speak of the probable or standard error of our estimate without further qualifications.

The method adopted is as follows: we estimate the frequency p of the gene **c** in the general population. We make out a pedigree showing the descent of the gene **h** in each pedigree, without reference to our knowledge concerning **c**. In doing so we have assumed that III 2 of fig. 1 and II 4 of fig. 3 were $\frac{+}{\mathbf{h}}$, and not mosaic. As they had seven haemophilic and only two normal sons, this seems to be justified. We have also neglected the possibility of illegitimacy. This would only be relevant in considering the daughters of a haemophilic man. Only two such occur in our pedigrees, and both proved their legitimacy by bearing haemophilic sons. Mutation at the **c** locus, if it occurs at all, is certainly too rare to be of any importance. So is non-disjunction.

Taking the pedigree of haemophilia as given, we determine:

1. $P(x, p)$. The probability, as a function of x and p, that, given that the first observed colour-blind man in the pedigree was actually colour-blind, all other relevant males in the pedigree possessed the type of colour vision which they actually did.

2. $P(\frac{1}{2}, p)$. The same probability for $x = \frac{1}{2}$, that is to say the probability of obtaining the observed result in the absence of linkage. If there have been no cross-overs, this is sufficient. If there have been, we must further calculate $P'(\frac{1}{2}, p)$, the probability of obtaining a result as favourable or more favourable to the hypothesis of linkage, in the absence of linkage.

3. $P(x, 0)$. The same probability for $p = 0$, i.e. neglecting the possibility that colour-blindness could have had a double origin.

4. $P(\frac{1}{2}, 0)$. The same possibility for $x = \frac{1}{2}$, $p = 0$, and $P'(\frac{1}{2}, 0)$ in case crossing-over has occurred.

The last is the easiest to calculate, and the first the hardest, but in practice it will be found that $P(x, 0)$ and $P'(\frac{1}{2}, 0)$ are sufficiently accurate. We shall see that all the values of $P(x, p)$ approximate very closely to $x^k(1-x)^{n-k}$, where n and k are not necessarily integers. We may say that we have tested

n ova and found k cross-overs. This simplified statement is sufficiently accurate for our data, but would not be so in general.

On multiplying the values of P for the six pedigrees we obtain a cumulative value. The cumulative value of $P'(\frac{1}{2}, p)$ gives us the probability that the data which appear to prove the linkage could really be due to sampling error. The assumption is involved that colour-blindness shows Mendelian segregation. Hogben (1932) has shown that this is very approximately true.

The cumulative value of $P(x, p)$ enables us to estimate x. The estimate is, however, as we shall see, extremely rough.

THE FREQUENCY OF DEUTERANOPIA IN THE GENERAL POPULATION

The colour-blindness in all six pedigrees here considered is deuteranopia. Data as to the frequency of protanopia and deuteranopia are given by Waaler (1927) for Oslo, v. Planta (1928) for Basle, Schmidt (1936) for Berlin, and Wright (unpublished) for London. Dr. Wright, who most kindly permits us to use his results, suspects that they may be a little high, as some of them were obtained from volunteers who may have had special reasons for desiring to be tested. The London value of $1{\cdot}20 \pm 0{\cdot}30$ % is well within the limits of sampling error of the overall value of $1{\cdot}42 \pm 0{\cdot}097$ %, and we shall take $p = 0{\cdot}0142$. It will be seen that the correction for p is actually negligible, but it will not always be so if the work begun in this paper is carried on.

TABLE I—PERCENTAGE FREQUENCY OF COLOUR-BLINDNESS IN MALES

	Waaler	v. Planta	Schmidt	Wright	Total
Number tested	9049	2000	6863	1338	19,250
Protanopes	0·88	1·60	1·09	1·27	1·01
Deuteranopes	1·03	1·50	1·97	1·20	1·42

PEDIGREE A (FIG. 1)

We assume that III 2 is heterozygous, though she is possibly a mosaic. If so, less weight is to be attached to the fact that V 5 is not a deuteranope; how much less weight is uncertain. We also know that IV 8 is heterozygous for haemophilia, since she had a haemophilic son, but we do not know the genotype of IV 7. We have now to determine P, given that IV 9 is a deuteranope, and that neither V 5 nor V 8 are deuteranopes.

It is at once clear that $P(\frac{1}{2}, 0) = \frac{9}{16}$. For if no information is derivable from linkage the probability that a deuteranope's sister is herself $\frac{+}{\mathrm{c}}$ is $\frac{1}{2}$,

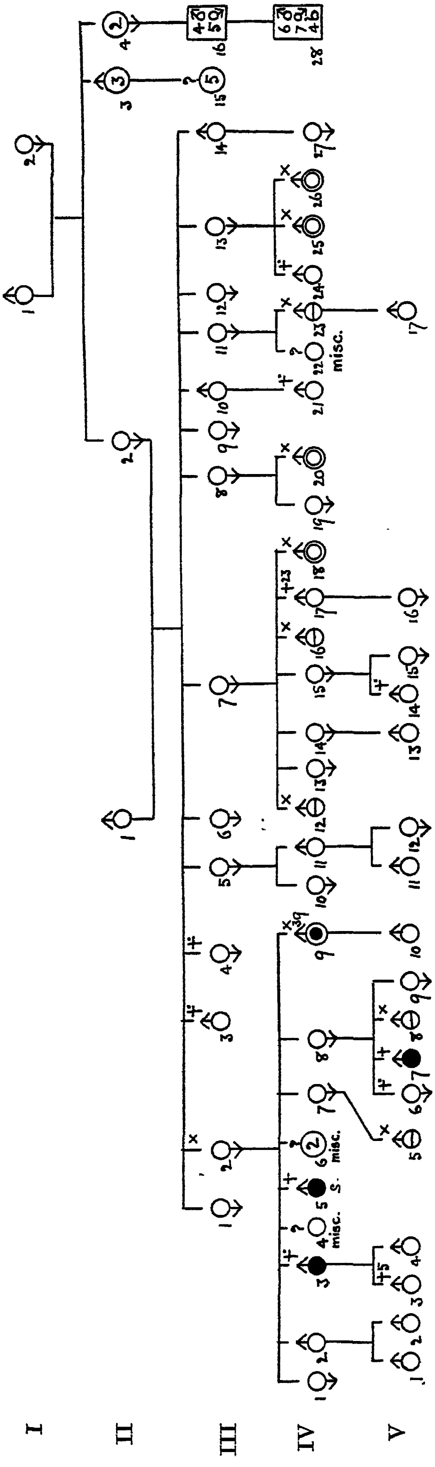

FIG. 1—Pedigree A. ⊙ haemophilic deuteranope; ● haemophilic; ◎ deuteranopic; ⊖ normal colour vision; × seen; + died in infancy.

and hence the probability that her only son, or only observed son, is a deuteranope is $\frac{1}{4}$. The probability that two such sons are not deuteranopes is therefore $(\frac{3}{4})^2$.

To calculate $P(x, 0)$ we note that III 2 is either $\frac{++}{\text{c h}}$ or $\frac{+\text{h}}{\text{c}+}$. The respective probabilities of these two genotypes are clearly y and x. If she was $\frac{++}{\text{c h}}$ the further probability that IV 8 is $\frac{++}{\text{c h}}$ is y, for she is known to be $\frac{+}{\text{h}}$, and the further probability that her non-haemophilic son is a non-deuteranope is y, making a cumulative probability of y^3 for this, the most probable causal nexus between IV 9 and V 8.

TABLE II—PROBABILITIES OF DIFFERENT CONNEXIONS OF V 8 AND V 5 WITH IV 9 (EXPLANATION IN TEXT)

V 8(++)	IV 8		III 2		IV 7		V 5(++)
			$\frac{++}{\text{c h}}$	y	$\frac{++}{++}$	$\frac{1}{2}y$	1
y	$\frac{++}{\text{c h}}$	y			$\frac{++}{+\text{h}}$	$\frac{1}{2}x$	1
1	$\frac{++}{+\text{h}}$	x			$\frac{++}{\text{c}+}$	$\frac{1}{2}x$	$\frac{1}{2}$
					$\frac{++}{\text{c h}}$	$\frac{1}{2}y$	y
			$\frac{+\text{h}}{\text{c}+}$	x	$\frac{++}{++}$	$\frac{1}{2}x$	1
y	$\frac{++}{\text{c h}}$	x			$\frac{++}{+\text{h}}$	$\frac{1}{2}y$	1
1	$\frac{++}{+\text{h}}$	y			$\frac{++}{\text{c}+}$	$\frac{1}{2}y$	$\frac{1}{2}$
					$\frac{++}{\text{c h}}$	$\frac{1}{2}x$	y

The various possibilities are scheduled in Table II. The first and last columns give the probability that two sons should have been born of mothers of the genotype given in the adjacent column. The second and fourth columns give the probability that a woman of the genotype considered should have been born of a mother of the genotype given in the central column. The central column gives the probability that a woman of that genotype should have been the mother of IV 9. Thus if III 2 is $\frac{+\text{h}}{\text{c}+}$ which has a probability x, then IV 7 is $\frac{+\,+}{+\,\mathrm{c}}$ with a further probability $\frac{1}{2}y$, and V 5 is + + with a further probability $\frac{1}{2}$.

Hence we find:

$$\begin{aligned} P(x,\,0) &= (y^2+x)\,y(\tfrac{1}{2}y+\tfrac{1}{2}x+\tfrac{1}{4}x+\tfrac{1}{2}y^2)+(y+xy)\,x(\tfrac{1}{2}x+\tfrac{1}{2}y+\tfrac{1}{4}y+\tfrac{1}{2}xy) \\ &= \tfrac{1}{4}(4y^4+8xy^3+17x^2y^2+7x^3y) \\ &= y^2+\tfrac{7}{4}x^2y+\tfrac{3}{2}x^2y^2 \\ &= y^2\left(1+\frac{13x^2}{4y^2}-\frac{19x^3}{4y^3}+\frac{25x^4}{4y^4}\cdots\right). \end{aligned}$$

Hence the leading term is y^2, and $n = 2$, $k = 0$.

To calculate $P(\frac{1}{2},\, p)$ we note that the only possible second source of deuteranopia is the husband of III 2. The probability that he was normal is $1-p$. If so the value of P is $\frac{9}{16}$, as found above. The probability that he was a deuteranope is p. If so his daughters are equally likely to be $\frac{+}{\mathrm{c}}$ or $\frac{\mathrm{c}}{\mathrm{c}}$. Hence the probability that V 5 is a deuteranope is $\frac{3}{4}$, and similarly for V 8. The probability that neither is a deuteranope is $\frac{1}{16}$. Hence

$$\begin{aligned} P'(\tfrac{1}{2}, p) = P(\tfrac{1}{2}, p) &= \tfrac{9}{16}(1-p)+\tfrac{1}{16}p \\ &= \frac{9-8p}{16} \\ &= \left(1-\frac{8p}{9}\right)P(\tfrac{1}{2},\, 0). \end{aligned}$$

Thus in this case the allowance for a secondary source diminishes $P(\frac{1}{2}, x)$ and thus increases the evidence for linkage. In other pedigrees it has the opposite effect. No further evidence would have been gained had the colour vision of IV 7 and IV 8 been examined, for, since each produced a son with normal vision, neither can have been $\frac{\mathrm{c}}{\mathrm{c}}$. An examination of IV 1 would, however, have given a little more information.

To calculate $P(x, p)$ we note that if the husband of III 2 was normal the analysis of Table II holds good. If he was deuteranopic the corresponding probabilities are given in Table III.

In this case the term contributed to $P(x, p)$ is

$$p[x^2y(\tfrac{1}{4}y+\tfrac{1}{2}x^2)+x^2y(\tfrac{1}{4}x+\tfrac{1}{2}xy)] = \tfrac{1}{4}px^2y(3x+y).$$

So $P(x,\,p) = \frac{1}{4}(1-p)\,(4y^4+8xy^3+17x^2y^2+7x^3y)+\frac{1}{4}p(x^2y^2+3x^3y)$

$$= \tfrac{1}{4}(4y^4+8xy^3+17x^2y^2+7x^3y)-p(y^4+2xy^3+4x^2y^2+x^3y)$$

$$= y^2+\tfrac{7}{4}x^2y+\tfrac{3}{2}x^2y^2-p(y^2+x^2y+2x^2y^2)$$

$$= (1-p)\left(y^2+\frac{7-4p}{4-4p}x^2y+\frac{3-4p}{2-2p}x^2y^2\right).$$

TABLE III—PROBABILITIES OF DIFFERENT CONNEXIONS OF V 8 AND V 5 WITH IV 9 IF III 2'S HUSBAND WAS A DEUTERANOPE

V 8(++)	IV 8		III 2		IV 7		V 5(++)
0	$\frac{\mathrm{c}+}{\mathrm{c\,h}}$	y	$\frac{++}{\mathrm{c\,h}}$	y	$\frac{\mathrm{c}+}{++}$	$\frac{1}{2}y$	$\frac{1}{2}$
x	$\frac{\mathrm{c}+}{+\mathrm{h}}$	x			$\frac{\mathrm{c}+}{+\mathrm{h}}$	$\frac{1}{2}x$	x
					$\frac{\mathrm{c}+}{\mathrm{c}+}$	$\frac{1}{2}x$	0
					$\frac{\mathrm{c}+}{\mathrm{c\,h}}$	$\frac{1}{2}y$	0
0	$\frac{\mathrm{c}+}{\mathrm{c\,h}}$	x	$\frac{+\mathrm{h}}{\mathrm{c}+}$	x	$\frac{\mathrm{c}+}{++}$	$\frac{1}{2}x$	$\frac{1}{2}$
x	$\frac{\mathrm{c}+}{+\mathrm{h}}$	y			$\frac{\mathrm{c}+}{+\mathrm{h}}$	$\frac{1}{2}y$	x
					$\frac{\mathrm{c}+}{\mathrm{c}+}$	$\frac{1}{2}y$	0
					$\frac{\mathrm{c}+}{\mathrm{c\,h}}$	$\frac{1}{2}x$	0

Hence the values of n and k are unaltered. Since the multiplication of $P(x,\,p)$ by a quantity independent of x does not affect the estimation of x, the sole effect of not neglecting p is to multiply the coefficient of x^2 by $1+\frac{3p}{7(1-p)}$ or 1·006, and that of x^2y^2 by $1-\frac{p}{3(1-p)}$ or 0·995. These corrections are quite negligible.

However, had V 5 been a deuteranope, the leading terms in $P(x,\,p)$ would have been $xy-py^2$. Since, as we shall see, x is a small quantity of the same order as p, though probably about three times as large, the correction would have been very important. If enough pedigrees are investigated such a case will probably arise, and a method has therefore been devised which is capable of dealing with it.

It is obvious that, in this case, we could calculate $P(x,\,p)$ directly, and derive the other three values of P by substitution. However, their direct evaluation is instructive, and also furnishes a useful check.

If III 2 was a mosaic we can readily obtain maximal values for P. Completely neglecting IV 7 and V 5, we find

$$P(x,\,0) = y + 2x^2y,$$

$$P(\tfrac{1}{2},\,0) = \tfrac{3}{4}.$$

If III 2 is a mosaic, the true values lie between these and those found earlier.

PEDIGREE B (FIG. 2)

To avoid circumlocution we have redrawn the relevant parts of pedigree B, and inserted some additional individuals in fig. 2*a*. Given the pedigree of haemophilia and the fact that M is a deuteranope, we have to calculate the probability P that I, L and N are deuteranopes, while E, F, G, H and K are not. Since α, β, γ, ζ and η all had haemophilic sons, it is clear that they are, or were $\frac{+}{\mathbf{h}}$, whilst ϵ may have been so. It may be remarked that the value of P is the same, from whichever of the haemophilic deuteranopes we start our calculation.

To calculate $P(x,\,p)$ we note that if there was only one source of deuteranopia, D must have been a deuteranope. Hence η is $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$ which has a probability y, and the further probability that N is a deuteranope is also y, giving a cumulative probability y^2. Since D was a deuteranope, the probability that β was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$ is y, the probability that she was $\frac{+\;\mathbf{h}}{\mathbf{c}\;+}$ is x. The cumulative probabilities of these two contingencies are thus y^3 and xy^2.

If β was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$, the probability that her three sons E, F and G are non-deuteranopes is y^3, the same probability for K being $\frac{1}{4}(4y^2 + 5xy + 3x^2)$ as found in the similar case in pedigree A. Further, β must have received both **c** and **h** from α. The probability that α was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$ is therefore y, the probability that she was $\frac{+\;\mathbf{h}}{\mathbf{c}\;+}$ is x. Since γ is known to have been $\frac{+}{\mathbf{h}}$, the further probability that she was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$ is $y^2 + x^2$. (She cannot have been $\frac{+\;\mathbf{h}}{\mathbf{c}\;+}$ if β was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$.) Given that γ was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$, the further probability that H and I were as found

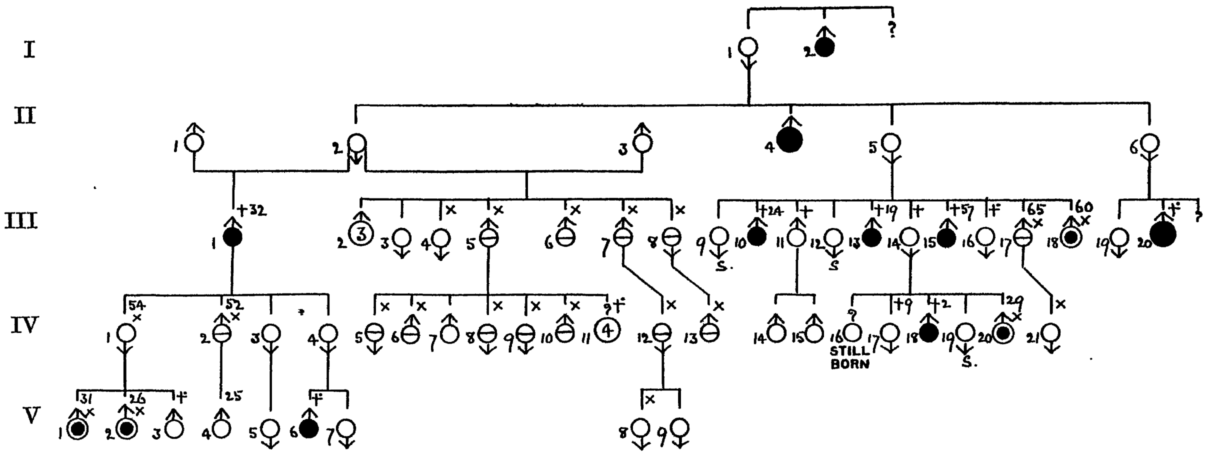

FIG. 2

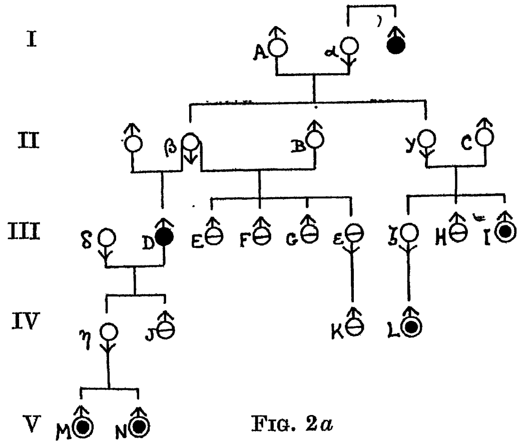

FIG. 2*a*

FIG. 2—Pedigree B. ● haemophilic; ⊙ haemophilic deuteranope, ⊖ normal colour vision; × seen; + died in infancy.

is y^2. The probability that L is a deuteranope is also y^2. Thus the cumulative probability from β onwards is

$$\tfrac{1}{4}y^7(4y^2+5xy+3x^2)\,(y^2+x^2),$$

and the contribution to $P(x,\,0)$ corresponding to the contingency that β was $\dfrac{+\,+}{\mathbf{c}\;\mathbf{h}}$ is

$$\tfrac{1}{4}y^{10}(4y^2+5xy+3x^2)\,(y^2+x^2).$$

If, however, β was $\dfrac{+\;\mathbf{h}}{\mathbf{c}\;+}$, the probability that E, F and G were as found is x^3. The further probability that K was as found is $\tfrac{1}{4}(3y^2+7xy+2x^2)$, the proof being as in pedigree A. Further, since β received **c** and **h** from different parents, A must have been **c**+ and $\alpha\,\dfrac{+\,+}{+\;\mathbf{h}}$. Hence γ was $\dfrac{+\;\mathbf{h}}{\mathbf{c}\;+}$. The probability that H and I were as found is x^2, the probability that L was as found xy. For ζ must have been $\dfrac{+\,+}{\mathbf{c}\;\mathbf{h}}$, as she was heterozygous for both genes. Hence the cumulative probability from β onwards is $\tfrac{1}{4}x^6y(3y^2+7xy+2x^2)$, and the contribution to $P(x,\,0)$ is

$$\tfrac{1}{4}x^7y^3(3y^2+7xy+2x^2)\,(y+x)^2,$$

the last term being added for the sake of homogeneity. Hence

$$\begin{aligned}P(x,\,0) &= \tfrac{1}{4}y^{10}(4y^2+5xy+3x^2)\,(y^2+x^2)+\tfrac{1}{4}x^7y^3(3y^2+7xy+2x^2)\,(y+x)^2\\ &= \tfrac{1}{4}(4y^{14}+5xy^{13}+7x^2y^{12}+5x^3y^{11}+3x^4y^{10}+3x^7y^7+13x^8y^6\\ &\qquad\qquad +19x^9y^5+11x^{10}y^4+2x^{11}y^3)\\ &= 1-\tfrac{51}{4}x+\tfrac{153}{2}x^2-\tfrac{1145}{4}x^3+\ldots\\ &= y^{\frac{51}{4}}\left(1+\frac{51x^2}{32y^2}-\frac{45x^3}{64y^3}+\ldots\right),\end{aligned}$$

$$n = 12\tfrac{3}{4},\ k = 0.$$

$$P(\tfrac{1}{2},\,0) = 3^2\times 2^{-13}.$$

It is to be noted that $y^{\frac{51}{4}}$ is a far better approximation than $1-\frac{51}{4}x$. Since $n = 12\frac{3}{4}$ we may say that the genes **c** and **h** have had $12\frac{3}{4}$ opportunities of separating, and have taken none of them. The steps in the pedigree, each of which contributes unity to n, are:

Mη, ηN, Dβ, βE, βF, βG, $\beta\alpha$, $\alpha\gamma$, γH, γI, $\gamma\zeta$ and ζL.

The two steps $\beta\epsilon$, ϵK contribute $\frac{3}{4}$ between them. We can at once apply this simple method to any given pedigree, and thus evaluate n and k. The residual terms such as $\frac{51}{32}x^2y^{\frac{43}{4}}$ represent contingencies involving crossing-over on two or more occasions. They are negligible when x is small, but essential for the calculation of $P(\frac{1}{2}, 0)$. In a large pedigree it may therefore be most convenient to calculate n and $P(\frac{1}{2}, 0)$ separately.

The calculation of $P(\frac{1}{2}, p)$ and $P(x, p)$ is somewhat more complicated, and the extra accuracy obtained turns out to be negligible. It is, however, necessary to demonstrate this fact, and the method demonstrated will be applicable in cases where the correction can no longer be neglected. Five contingencies would give a second source of deuteranopia in the pedigree:

1. δ produced a **c**+ gamete from which η was formed.
2. B was **c**+.
3. A was **c**+ and α was $\frac{+}{\mathbf{c}}\left(\text{either } \frac{+\,+}{\mathbf{c\,h}} \text{ or } \frac{+\,\mathbf{h}}{\mathbf{c}\,+}\right)$.
4. α was $\frac{\mathbf{c}+}{\mathbf{ch}}$.
5. C was **c**+.

Each of these contingencies has an *a priori* probability p. Further, any two or more of them may be true simultaneously, except (3) and (4). These are excluded because β and γ had sons with normal vision and were therefore not $\frac{\mathbf{c}}{\mathbf{c}}$. We shall neglect these multiple contingencies, which contribute to the coefficient of p^2 in the expansion of P. Since p^2 is much smaller than the standard error of p, this is clearly justifiable.

It is most convenient to calculate $P(\frac{1}{2}, p)$ and $P(x, p)$ separately, since we are only interested in the leading terms in the expansion of the latter. To calculate $P(\frac{1}{2}, p)$ we multiply $P(\frac{1}{2}, 0)$ by $(1-5p)$ and add terms corresponding to each of the five contingencies. These are as follows:

1. Since J is not colour-blind, the probability must be halved. For if δ had been $\frac{+}{\mathbf{c}}$, the chance of his being so would be $\frac{1}{2}$. On this hypothesis it is no longer sure that D was colour-blind, and it is best to begin the calculation at the other end of the pedigree, that is to say to ask "Given that I is colour-blind, and that δ contributed a **c**+ gamete to η, what is the probability that the various individuals had the type of colour vision found, neglecting the information supplied by the pedigree of haemophilia?" On this hypothesis the value of $P(\frac{1}{2}, 0)$ would be calculated as follows: γ was $\frac{+}{\mathbf{c}}$. Hence the probability that H and L are as found is 2^{-3}. The probability that

β was $\dfrac{+}{+}$ is 2^{-2}, the probability that she was $\dfrac{+}{\mathrm{c}}$ is 3×2^{-2}. If β was $\dfrac{+}{+}$, the probability that E, F, G and K are as found is 1. And since D was +, the probability that M and N were colour-blind is 2^{-2}. If however β was $\dfrac{+}{\mathrm{c}}$ the probability that E, F, G and K are normal is 3×2^{-5}. The further probabilities that D was + and **c** are each $\frac{1}{2}$. If D was +, the probability that M and N are colour-blind is $\frac{1}{4}$. If D was **c**, the probability is 1. Hence on this hypothesis

$$\begin{aligned} P(\tfrac{1}{2},\,0) &= 2^{-3}[2^{-2}\times 2^{-2}+3\times 2^{-2}\times 3\times 2^{-5}\times 2^{-2}(2^{-2}+1)] \\ &= 173\times 2^{-14}. \end{aligned}$$

So the contribution to $P(\frac{1}{2}, p)$ on this contingency is $173\times 2^{-15}p$.

2. The probability that a colour-blind man B and his heterozygous wife β should have a normal daughter whose only son is normal is $\frac{1}{4}$, as opposed to $\frac{3}{4}$ if B was normal. The contribution is therefore $3\times 2^{-13}p$.

3. The probability that β and γ were both heterozygous is now $\frac{1}{4}$ instead of $\frac{3}{4}$, so the contribution is $3\times 2^{-13}p$.

4. The probability that γ was heterozygous is now 1 instead of $\frac{3}{4}$, and the contribution is therefore $3\times 2^{-11}p$.

5. The probability, given that γ was heterozygous, that L is colour-blind is now $\frac{3}{4}$ instead of $\frac{1}{4}$, so the contribution is $3^3\times 2^{-13}p$.

Hence $P(\frac{1}{2}, p)$

$$\begin{aligned} &= (1-5p)\,9\times 2^{-13}+p(173\times 2^{-15}+3\times 2^{-13}+3\times 2^{-13}+3\times 2^{-11}+27\times 2^{-13}) \\ &= 9\times 2^{-13}+173\times 2^{-15}p \\ &= 9\times 2^{-13}\left(1+\frac{173p}{36}\right). \end{aligned}$$

Thus $P(\frac{1}{2}, 0)$ must be multiplied by 1·0673.

In calculating $P(x, p)$ we need only consider the changes in the first three terms, namely $y^{14}+\frac{5}{2}xy^{13}+\frac{7}{4}x^2y^{12}$, in the homogeneous expansion of $P(x, 0)$, since it will be shown later that the third term and *a fortiori* later terms are negligible. As above, each contingency contributes a term to be added to $(1-5p)\,P(x, 0)$.

1. Since J is + +, the term must be multiplied by $\frac{1}{2}$. If η is $\dfrac{\mathrm{c}\,+}{\mathrm{c}\,\mathrm{h}}$, M and N must have been deuteranopes. The probability is $y^{-2}P(x, 0)$. If η was $\dfrac{\mathrm{c}\,+}{+\,\mathrm{h}}$, the probability that M and N are deuteranopes is x^2. Further, D was not a deuteranope, which involves a further probability of x if β was $\dfrac{+\,+}{\mathrm{c}\,\mathrm{h}}$,

and a power of x if she was $\frac{+\ \mathbf{h}}{\mathbf{c}\ +}$. Hence the probability that η was $\frac{\mathbf{c}\ +}{+\ \mathbf{h}}$ is negligible, and the contribution is $\frac{1}{2}y^{-2}P(x,\,0)\,p$ or

$$\tfrac{1}{2}(y^{12}+\tfrac{5}{4}xy^{11}+\tfrac{7}{4}x^2y^{10}+\ldots)\,p.$$

Multiplying by $(y+x)^2$ for the sake of homogeneity, this becomes

$$(\tfrac{1}{2}y^{14}+\tfrac{13}{8}xy^{13}+\tfrac{21}{8}x^2y^{12}+\ldots)\,p.$$

2. If β was $\frac{+\ \mathbf{h}}{\mathbf{c}\ +}$, the probability has a leading term x^7y^7 and is negligible. If β was $\frac{+\ +}{\mathbf{c}\ \mathbf{h}}$, the probability that ϵ and K have normal vision is $\frac{1}{4}(y^2+xy+2x^2)$ instead of $\frac{1}{4}(4y^2+5xy+3x^2)$. So the contribution to $P(x,\,p)$ is

$$(\tfrac{1}{4}y^{14}+\tfrac{1}{4}xy^{13}+\tfrac{3}{4}x^2y^{12}+\ldots)\,p.$$

3. If A was **c**+, then, since β and γ were not $\frac{\mathbf{c}+}{\mathbf{c}\,\mathbf{h}}$, they must have been $\frac{\mathbf{c}\ +}{+\ \mathbf{h}}$. As shown above the probability has a leading term x^7y^7 and is negligible.

4. β must have been $\frac{+\ +}{\mathbf{c}\ \mathbf{h}}$, so must γ. That is to say the probability that γ was $\frac{+\ +}{\mathbf{c}\ \mathbf{h}}$ is raised from y^2+x^2 to unity. So we must substitute $(y+x)^2$ for y^2+x^2 in the expression for P. The contribution is thus

$$(y^{14}+\tfrac{13}{4}xy^{13}+\tfrac{17}{4}x^2y^{12}+\ldots)\,p.$$

5. Assuming that γ was $\frac{+\ +}{\mathbf{c}\ \mathbf{h}}$ and C was **c**+, the probability that L is a deuteranope is raised from y^2 to y^2+xy+x^2. The probability that γ was $\frac{+\ \mathbf{h}}{\mathbf{c}\ +}$ is negligible. So the contribution is

$$(y^{14}+\tfrac{9}{4}xy^{13}+4x^2y^{12}+\ldots)\,p.$$

Hence

$$P(x,p)=(1-5p)\,(y^{14}+\tfrac{5}{4}xy^{13}+\tfrac{7}{4}x^2y^{12}+\ldots)+p(\tfrac{11}{4}y^{14}+\tfrac{59}{8}xy^{13}+\tfrac{93}{8}x^2y^{12}+\ldots)$$

$$=\frac{4-9p}{4}\left(y^{14}+\frac{10+9p}{8-18p}xy^{13}+\frac{14+23p}{8-18p}x^2y^{12}+\ldots\right)$$

$$=\left(1-\frac{9p}{4}\right)y^{14-\frac{10+9p}{8-18p}}\left[1+\frac{204-558p-1171p^2}{8(4-9p)^2}\frac{x^2}{y^2}+\ldots\right]$$

or, neglecting powers of p,

$$=\left(1-\frac{9p}{4}\right)y^{\frac{51}{4}-\frac{63p}{16}}\left(1+\frac{51+115p}{32}\frac{x^2}{y^2}+\ldots\right).$$

Hence n is decreased from 12·75 to 12·695. It will be seen later that this has a negligible effect on the estimate of x.

RIDDELL'S PEDIGREE

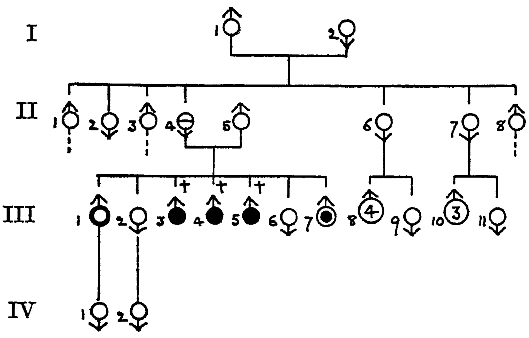

FIG. 3—Riddell's Pedigree. ● haemophilic; ⊙ colour-blind haemophilic; ◎ colour-blind; ⊖ normal colour vision.

Here we have the only case of crossing-over in our material. If II 4 is $\frac{+}{\mathrm{h}}$, then, whether she is $\frac{+\ +}{\mathrm{c\ h}}$ or $\frac{+\ \mathrm{h}}{\mathrm{c}\ +}$, the value of $P(x, 0)$ that is to say, the probability, given that III 7 is colour-blind, that III 1 should also be colour-blind is $2xy$. There is no correction for a second source of colour-blindness, since II 4 is not colour-blind. So

$$P\,(x,\,p) = 2xy,$$

$$P(\tfrac{1}{2}, p) = \tfrac{1}{2},$$

$$n = 2, \quad k = 1.$$

In this case, however, the appropriate function is $P'(\frac{1}{2}, p)$, the probability that a result as favourable to the hypothesis of linkage, or more so, should arise from random sampling. As the observed result is as unfavourable as possible, it follows that

$$P'(\tfrac{1}{2}, p) = 1.$$

Unfortunately there is, at least in the present state of our knowledge, the possibility that II 4 is a mosaic who was originally $\frac{+\ +}{\mathrm{c}\ +}$ but of whom a portion has mutated to $\frac{+\ +}{\mathrm{c\ h}}$, or $\frac{+\ \mathrm{h}}{\mathrm{c}\ +}$. Both types of tissue would be represented in her ovaries. We consider such a possibility unlikely, but to neglect it would be intellectually dishonest. Hence an alternative explanation, though not, we think, a probable one, can be given for the apparent case of crossing-over. Crossing-over will not be conclusively demonstrated until it occurs in the gametes of a woman who could not be a mosaic.

BIRCH'S PEDIGREE

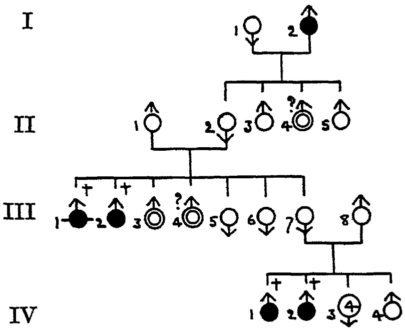

FIG. 4—Birch's Pedigree. ● haemophilic; ◎ colour-blind; -●- haemophilic, normal colour vision.

Here our only certain information regarding vision concerns III 1 and III 3. $P(x, 0)$ is calculated as follows: given that III 3 is colour-blind, the probability that II 2 is $\frac{+\ +}{\mathbf{c}\ \mathbf{h}}$ is y, the probability that she is $\frac{+\ \mathbf{h}}{\mathbf{c}\ +}$ is x. Hence the probability that III 1 was not colour-blind is y^2+x^2. So

$$P(x, 0) = y^2+x^2,$$

$$P(\tfrac{1}{2}, 0) = \tfrac{1}{2}.$$

If and when it is established that III 4 is colour-blind, these values will be diminished to y^3+x^3 and $\frac{1}{4}$. If II 4 is colour-blind, I 1 must have been $\frac{+\ +}{\mathbf{c}\ +}$; so II 2 was $\frac{+\ \mathbf{h}}{\mathbf{c}\ +}$ and $P(x, 0) = y^2$ or y^3. There is here no correction for a second source of colour-blindness, since II 2 had both colour-blind and normal sons and must be heterozygous for **c**. We therefore find

$$P(x, p) = y^2+x^2,$$

$$P(\tfrac{1}{2}, p) = \tfrac{1}{2},$$

$$n = 2,\quad k = 0,$$

with the proviso that the values of P will probably be reduced by further information.

MADLENER'S PEDIGREE

Madlener (1928) published the first pedigree in which linkage between colour-blindness and haemophilia is probable. He has kindly supplemented it by further information. The relevant part of this pedigree is given in fig. 5. I 1 had a number of haemophilic relatives. I 1, III 2, and III 4 are described as colour-blind (*rot-grünblind,* presumably deuteranopic) in Madlener's paper, and the latter two were tested at the time when it was written. IV 3 was at that time too young to test.

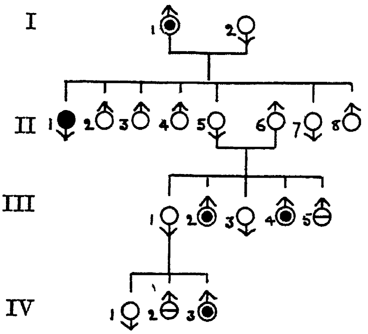

FIG. 5—Madlener's Pedigree. ⊙ Colour-blind haemophilic; ⊖ normal colour vision; ● haemophilic.

In answer to our enquiry Madlener consulted III 1, whom he describes as having great *Familiensinn.* She gave him the following further information. III 5 and IV 2 have normal colour vision, and IV 3, who is still alive and has had no severe haemorrhage since 1928, is definitely colour-blind. She knows of no colour-blind members of the family other than the haemophilics. While her information does not carry so much weight as that concerning III 2 and III 4 who are known to have been examined by a colleague of Dr. Madlener's, we see no reason to reject it.

The most remarkable feature of the pedigree is the haemophilic girl II 1, whose case is carefully discussed by Madlener. Unfortunately he had not personally examined her. He concluded that she was a genuine haemophilic. Now her mother, I 2, had four normal sons and three normal brothers. Further, her father, maternal grandfather, two maternal uncles, and five sons of two sisters were normal. The probability of this, if she was heterozygous for haemophilia, is $5{\cdot}9 \times 2^{-15}$, or 0·00009. If she was homozygous, the chance that she produced an **h** gamete is about 0·00002. Lenz suggested to Madlener that she was a pseudo-hermaphrodite, and really a male. This involves the fantastically unlikely coincidence of the transference of haemophilia from father to son with gross anatomical abnormality in the son. It seems safest to conclude either that **h** is here dominant over its normal allelomorph, or that the case was incorrectly diagnosed.

$P(x, 0)$ is calculated as follows: II 5 was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$. The probability that III 2, III 4, and III 5 were as found is y^3. Further III 1, since she transmitted both **c** and **h**, and could only have got them from her mother, must be $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$. This has a further probability y. The further probability that her two sons IV 2 and IV 3 are as found is y^2. Thus

$$P(x, 0) = y^6,$$

$$P(\tfrac{1}{2}, 0) = 2^{-6}.$$

The expressions are very simple when, as in this case, we have exact information about the common ancestor. The two possible secondary sources of colour-blindness are I 2 and II 6. If I 2 had contributed a **c**+ gamete to II 5, II 5 would have been $\frac{\mathbf{c}+}{\mathbf{c}\mathbf{h}}$, and III 5 would have been colour-blind. Hence the effect of considering I 2 is to multiply P by $(1-p)$. If II 6 was colour-blind, III 1, who is known to be $\frac{+}{\mathbf{h}}$ and $\frac{+}{\mathbf{c}}$, must be $\frac{\mathbf{c}+}{+\mathbf{h}}$, which has a probability x, since II 5 was $\frac{+\;+}{\mathbf{c}\;\mathbf{h}}$. The further probability that IV 2 and IV 3 are as found is x^2.

So
$$P(x, p) = (1-p)\,[(1-p)\,y^6 + px^3y^3]$$
$$= (1-p)^2 y^6 \left[1 + \frac{px^3}{(1-p)\,y^3}\right],$$
$$n = 6, \quad k = 0,$$

and
$$P(\tfrac{1}{2}, p) = (1-p)\,2^{-6}.$$

GREEN'S PEDIGREE

I have to thank the late Dr. C. V. Green for full information about this pedigree, which was compiled by him and published by Davenport (1930). The relevant part is given in fig. 6. I 2, who is III 7 of Davenport's pedigree, had a colour-blind brother, two colour-blind maternal uncles, and other colour-blind relatives, but no haemophilic relatives other than those shown. The colour-blindness appears to be deuteranopia. Let us first assume that I 2 was a double heterozygote, and not a mosaic. If so, since she was almost certainly $\frac{+\mathbf{h}}{\mathbf{c}+}$, she derived the gene **h** from a mutated paternal gamete, since her father was not haemophilic, and her colour-blindness came from her mother.

In this pedigree **c** and **h** are repelled. We ask, given the pedigree of haemophilia and the fact that II 3 is colour-blind, what is the probability that II 4, II 6, III 1 and III 5 are as found? Since I 1 and II 1 are not colour-blind a second source is excluded, and $P(x, p) = P(x, 0)$.

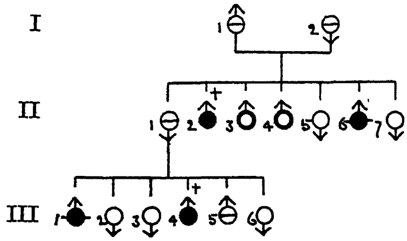

FIG. 6—Green's Pedigree. ◎ colour-blind; ● haemophilic; ⊖ normal colour vision; -●- haemophilic, normal colour vision.

The probability that I 2 is $\frac{+\mathbf{h}}{\mathbf{c}+}$ is y, the probability that she is $\frac{+\,+}{\mathbf{c}\,\mathbf{h}}$ is x. Hence the probability that II 4 and II 6 are as found is y^3 in the first case, x^3 in the second. If I 2 is $\frac{+\mathbf{h}}{\mathbf{c}+}$, the probability that II 1 is $\frac{+\,+}{+\,\mathbf{h}}$ is y, in which case III 1 and III 5 were certainly non-deuteranopic. The probability that II 1 is $\frac{+\,+}{\mathbf{c}\,\mathbf{h}}$ is x, and the further probability that III 1 and III 5 are not deuteranopic is xy. So if I 2 is $\frac{+\mathbf{h}}{\mathbf{c}+}$, the probability that III 1 and III 5 are as found is $y+x^2y$.

If on the other hand I 2 is $\frac{+\,+}{\mathbf{c}\,\mathbf{h}}$, the probabilities that II 1 is $\frac{+\,+}{\mathbf{c}\,\mathbf{h}}$ and $\frac{+\,+}{+\,\mathbf{h}}$ are y and x respectively, and the probability that III 1 and III 5 are non-deuteranopes is $x+xy^2$.

Hence
$$P(x, p) = P(x, 0) = y^3(y+x^2y)+x^3(x+xy^2)$$
$$= y^4+x^2y^4+x^4(1+y^2)$$
$$= y^4\left(1+\frac{x^2}{y^2}-\frac{2x^3}{y^3}+\dots\right)$$
and
$$n = 4,\ k = 0,$$
$$P(\tfrac{1}{2}, p) = P(\tfrac{1}{2}, 0) = 5\times 2^{-5}.$$

Let us suppose that I 2 is a mosaic of $\frac{+\;+}{\mathbf{c}\;+}$ and $\frac{+\mathbf{h}}{\mathbf{c}+}$ $\left(\text{or possibly } \frac{+\;+}{\mathbf{c}\;\mathbf{h}}\right)$. We cannot be sure that II 3 and II 4 were derived from the latter. Hence our value of P must be based on II 6, III 1 and III 5, though II 3 and II 4 give us the information that I 2 is heterozygous for **c**. In this case

$$\begin{aligned} P(x, p) &= y(y+x^2y)+x(x+xy^2) \\ &= y^4+2xy^3+4x^2y^2+2x^3y+x^4 \\ &= y^2+3x^2y^2+2x^3y+x^4, \end{aligned}$$

for $P(\frac{1}{2}, p) = 5\times 2^{-3}$, $n = 2$, $k = 0$. However, the former hypothesis seems much more probable.

SIGNIFICANCE OF THE DATA

Assuming that all doubtful cases were heterozygotes and not mosaics, the product of the five values of $P(\frac{1}{2}, 0)$ and one of $P'(\frac{1}{2}, 0)$ is $3^4\times 5\times 2^{-29}$, or $7{\cdot}54\times 10^{-7}$. The product of the values of $P(\frac{1}{2}, p)$ is this number multiplied by approximately $1+\frac{35p}{12}$ or $1{\cdot}041$, that is to say $7{\cdot}84\times 10^{-7}$. This number would be appreciably modified if the segregation of **c** in heterozygous mothers were abnormal. But it could hardly exceed 10^{-5}.

Let us next assume that all the doubtful women were mosaics, and, further, that all their non-haemophilic sons were derived from their non-mutated sectors. Both these hypotheses are most unlikely. But, even so, the product of the $P(\frac{1}{2}, 0)$ values is $3^3\times 5\times 2^{-25}$, or $4{\cdot}04\times 10^{-6}$. The true value lies somewhere between this and $7{\cdot}84\times 10^{-7}$, a good deal closer to the latter.

In other words, the probability that our finding of linkage is an error due to a run of luck is about one in a million. It is, moreover, difficult to frame any hypothesis other than linkage which will account for the results. In three pedigrees, A, B and Madlener's, the two genes are coupled; in two pedigrees, those of Birch and Green, they are repelled. This fact at once negates any theory as to a physiological connexion between the two diseases. It is, however, exactly what would be expected from analogy with animal genetics.

ESTIMATION OF THE FREQUENCY OF CROSSING-OVER

We shall now consider the estimation of x on the hypothesis that all small positive values have an equal *a priori* probability. We first assume that the three doubtful women were not mosaics. The product of the leading terms in the product of the six values of $P(x, p)$ is

$$x^{\Sigma k}(1-x)^{\Sigma(n-k)} = x(1-x)^{27{\cdot}75}.$$

If this is plotted against x we get a very skew frequency curve giving the probability that x lies between given limits. The modal or maximum likelihood value is $x = \frac{1}{28{\cdot}75} = 0{\cdot}035$. This result may, however, be rather misleading. The mean value is

$$\frac{\int_0^1 Px\,dx}{\int_0^1 P\,dx} = 0{\cdot}065.$$

This is the estimated probability that the next son of a doubly heterozygous woman to be examined will be the product of crossing-over. The median X is given by

$$\int_0^X P\,dx = \tfrac{1}{2}\int_0^1 P\,dx$$

and is equal to 0·057. That is to say, the frequency of recombination based on a large sample is as likely to exceed 5·7 % as to fall below it. The quartile values are 0·032 and 0·088, so this estimate is very uncertain.

We have next to determine the corrections due to including the terms in the expansion of P involving x^3 on the one hand, and p on the other. The product of the values of $P(x, 0)$ is

$$xy^{27{\cdot}75} + \tfrac{219}{32}x^3y^{25{\cdot}75} + \dots.$$

The mean is therefore increased from 0·0650 to 0·0685, with similar slight effects on the mode and median.

The correction for secondary sources is still more negligible. The estimation of x is not affected by the constant terms by which P is multiplied. Σn is reduced from 28·75 to 28·695. This raises the mean from 0·0650 to 0·0651. There is also a very slight change in the correction for x^3. It must however be remembered that in future work it may not be possible to neglect one or both of these corrections.

If all the three doubtful women were mosaics, and further if all their non-haemophilic sons were derived from non-mutated tissue, we should have $n = 23{\cdot}75$, $k = 0$. The modal value of x would be zero, the mean 3·88 %, and the median 2·76 %, with quartiles at 1·16 % and 5·45 %. It must, however, be remembered that somatic mosaics in man are very rare.

Cockayne (1933) records three only for conditions affecting the skin, and these were very probably due to chromosome elimination rather than gene mutation. Finally, even if all the women in question were mosaics, it is unlikely that all their non-haemophilic sons were derived from mutated tissue. There is therefore very little reason for lowering our estimates of x.

THE *A PRIORI* PROBABILITY

The calculations so far made other than the estimate based on maximum likelihood would be true if, and only if, the *a priori* probability of all small values of x were equally great. This is certainly not so. First consider a chromosome of genetical length l, that is to say in which the sum of the probabilities of crossing-over between a series of genes at short distances along the chromosome is l. Further, suppose that both genes and probabilities of crossing-over are evenly distributed along it. Then the distribution of x, the distance between two genes taken at random (which for small values, such as exclude double crossing-over, is equal to the distance between the genes), is

$$df = \frac{2(l-x)}{l^2}\,dx.$$

If l is of the order of unity, which is probably the case for the human X-chromosome, this will entail a correction equivalent to adding unity to the value of n, and the median estimate will be reduced from 0·057 to 0·053.

But actually the correction is probably larger, though its magnitude is unknown. If we consider the existing data on linkage in animals, we find that there are two fairly sharply contrasted types. The common type, illustrated by the female *Drosophila*, the mouse, rabbit, hen, etc., shows cross-over values ranging from nearly zero to about 50 %, and possibly exceeding this value in rare cases. In the other type, which is characteristic of the male *Drosophila*, and of both sexes in such polymorphic species as *Lebistes reticulatus* and *Paratettix texanus*, all, or almost all, cross-over values are less than 10 %. The evidence brought forward by Haldane (1936) suggests that man belongs to the first class, as do the four rodents which are the only other mammals in which linkage has been studied.

Now the chiasmata, whose genetical effect is crossing-over, always seem to be somewhat localized. That is to say, crossing-over is relatively rare in some regions of a chromosome. The genes may or may not be evenly distributed, but regions are generally found where genes are common, and crossing-over rare. Thus in the distal or "left-hand" end of the X-chromosome and the proximal regions of the autosomes of *Drosophila melanogaster* many genes are located, but crossing-over is very infrequent. And if two genes are taken at random, there is a considerable probability that both will lie in such a region. Hence cross-over values between pairs of genes taken at random show a peculiar distribution. There is a sharp maximum in the neighbourhood of zero, and another maximum somewhere between 45 and 50 % due to genes at considerable distances from one another. The curve then drops again to zero near 50 %, but this part of the curve is difficult to map, owing to large errors, due both to sampling and to differential viability.

Fig. 7 represents the distribution of 3828 map distances between 88 genes located in the X-chromosome of *Drosophila melanogaster*. It was kindly plotted for us by Mr. F. Minns from data given in Drosophila Information Service, corrected for unpublished results obtained by Dr. H. Grüneberg on the location of *roughest*. The distances were grouped by units. The drop between 1 and 2 units is perhaps accidental. The data for the autosomes, though less numerous, yield similar results.

Since cross-over values are very approximately equal to map distances for values less than 0·15, but do not exceed 0·50 when the map distance does so, it follows that the maximum in the neighbourhood of zero is correctly represented, while the second maximum is not. If $df = f(x)\,dx$ be the frequency distribution of cross-over values, and $P(x)$ is defined as above, we can answer the following question: "If the human data here given referred to *Drosophila melanogaster*, what would be the median cross-over value to be expected?"

The median value X is given by

$$\int_0^X P(x) f(x)\,dx = \tfrac{1}{2}\int_0^1 P(x) f(x)\,dx.$$

We obtain a value of $f(x)$ by smoothing. Mr. Minns has fitted a quintic to the points between $x = 0{\cdot}005$ and $0{\cdot}355$ on fig. 7.

If $z = 100x - 17{\cdot}5$, its equation is

$$f(z) = 72{\cdot}618 - 1{\cdot}7756z - 0{\cdot}17326z^2 + 0{\cdot}01634z^3 + 0{\cdot}0009737z^4 - 0{\cdot}00007408z^5.$$

The values given by the equation were used up to $x = 0{\cdot}15$. For higher values $f(x)$ was taken as constant, since as the result of double crossing-over it exceeds the value deducible from fig. 7. The inaccuracy so introduced is slight, as the area of the tail of the curve for $P(x) f(x)$ beyond $x = 0{\cdot}15$ is only 5·0 % of the total.

The modal value of x is about 2·5 %. The median is 4·7 %, with quartiles at 2·5 and 8·1 %. The curve is rendered skewer by the correction, and the estimates of x are all lowered. However, the change is less than the probable error. It is clear that the correction for *a priori* probability will become

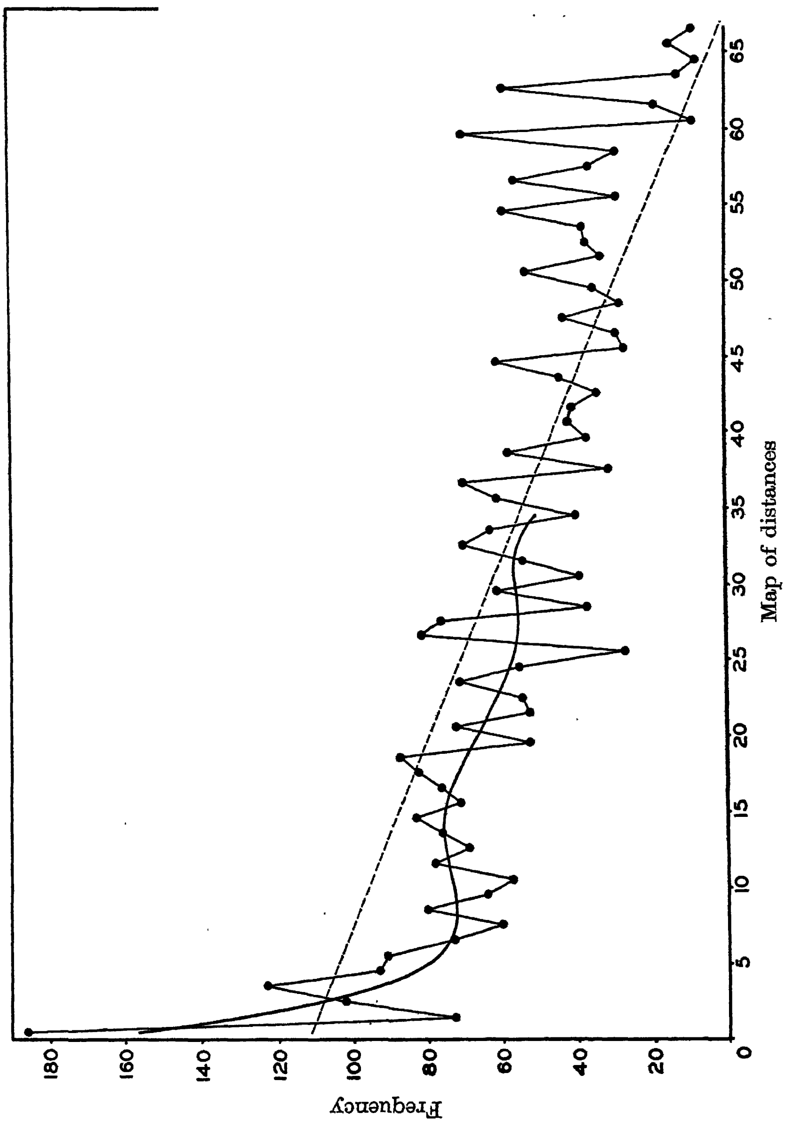

FIG. 7.—Frequencies of map distances between pairs of genes in the X-chromosome of *Drosophila melanogaster*. Dotted line, frequency distribution if genes were evenly distributed on the genetical map. Curve, quintic giving best fit.

negligible when enough pedigrees are available. Meanwhile we may take 5 % as a reasonable median value.

The correction for *a priori* probability is considerably larger if we neglect the possibly mosaic women, taking $n = 23{\cdot}75$, $k = 0$. For in this case the sharp maximum of $f(x)$ when x is zero is of much greater importance. The mode is zero, the median 1·9 %, or half its former value.

DISCUSSION

The linkage here investigated is so close that on quite a small amount of material it has been possible to demonstrate its existence without leaving grounds for reasonable doubt. If such close linkage is typical of the human species, the search for further cases will be an easier task than appeared probable at first sight, and its results will be of considerable practical value.

The present case has no prognostic application, since haemophilia can be detected before colour-blindness. If, however, to take a possible example, an equally close linkage were found between the genes determining blood group membership and that determining Huntington's chorea, we should be able, in many cases, to predict which children of an affected person would develop this disease, and to advise on the desirability or otherwise of their marriage.

Meanwhile, as a means to the mapping of the X-chromosome, it is desirable that all persons suffering from any disease showing sex-linked inheritance, and their brothers, should be examined for colour-blindness and, if possible, for anomalous trichromatism. Since about 8 % of males in north-western Europe show one or other of these defects, the search should be fruitful.

This research should lead, within a few years, to a determination of the approximate map distances of the loci of these genes from that of colour-blindness. Mapping would not be possible until another common sex-linked, or incompletely sex-linked, gene substitution has been discovered. The search for new immunological characters may be expected to reveal such a gene within the next generation.

Should Haldane's (1936) claim to have discovered a group of incompletely sex-linked genes be confirmed, it is also desirable that male cases of xeroderma pigmentosum, recessive epidermolysis bullosa dystrophica, etc., and their brothers should be similarly examined.

The theoretical method used in this paper has been unduly cumbrous. It has, however, been given in full for three reasons. In the first place it was necessary to show that certain conditions could be neglected. Secondly, the correction for a second source of colour-blindness will become important when a colour-blind man of an unexpected type as regards blood coagulation is discovered, and it is uncertain whether he is due to crossing-over or to a second source of colour-blindness. In Riddell's pedigree this uncertainty was avoided by testing the colour vision of the mother. Finally, the later terms in the expansion of $P(x, 0)$ will become important if, in the case of another sex-linked character, x turns out to be larger. Thus if there were 20 % of crossing-over between the loci of the genes for colour-blindness and anidrosis, $\frac{x^2}{y^2}$ would be 0·0625 instead of 0·0028, and would no longer be negligible.

The following simplified method may be used for the further investigation of the linkage between colour-blindness and haemophilia:

1. The pedigrees of haemophilia and colour-blindness are determined. In each case crossing-over is assumed not to have occurred unless there is evidence that it has occurred. A single source only of each abnormality is assumed.

2. A number, n, of gametes tested is determined by the following conventions: for each relationship between a doubly heterozygous mother and a child whose genotype is observed or deduced, n is increased by unity. The amount by which n is to be increased for daughters of double heterozygotes whose genotype is not inferable with certainty is a matter for calculation in individual cases. Examples occur in three of the pedigrees here considered.

3. A number, k_1, of undoubted cross-over gametes is similarly calculated. If there are cases which could be explained either by crossing-over or a second source of colour-blindness, numbers k_2, k_3, etc., of facultative cross-overs are calculated.

4. If k_2, k_3, etc. $= 0$, the probabilities regarding x are as if we had observed k_1 cross-overs in a sample of n. The estimate $\frac{k_1}{n}$ is therefore a modal value, and so long as k_1 is small will differ appreciably from the mean $\frac{k_1+1}{n+2}$, and the median.

5. If some of the apparent cross-overs are colour-blind, and a second source is possible, the likelihood of a given value of x is an expression of the form

$$P = (1-x)^{n-k_1-k_2-k_3\dots} x^{k_1} (x-p)^{k_2} (x-\tfrac{1}{2}p)^{k_3}\dots.$$

An example of the manner in which these latter terms might arise is given on p. 131. The estimates of x are to be obtained by determining the mode, mean, and median of this function.

6. Until more is known as to the circumstances in which haemophilia arises by mutation it is desirable to estimate x on two different samples. The first would include the progeny of all women who are certainly heterozygous. The second would exclude all persons who might have arisen from

a homozygous portion of the ovaries of a woman who was possibly a mosaic due to somatic mutation. If these two samples differ significantly, the second must be used for our estimation. Until such a difference is proved we can see no good reason to correct for the rather remote possibility of mosaicism.

7. Until n is of the order of 100 the estimates of x will be appreciably too high, owing to the fact that small values of x have a greater *a priori* probability than large.

Summary

Four new pedigrees are described in which cases both of colour-blindness and of haemophilia occur. The two pedigrees already published are considered, and the information concerning one of them is supplemented. In three of the six pedigrees haemophilia appears to have arisen afresh by mutation.

The genes for colour-blindness and haemophilia are closely linked. The probability that the results attributed to linkage could have arisen by sampling is less than 4×10^{-6}. So far only one case of crossing-over has occurred, and even this is not absolutely certain, as the mother of the man in question may possibly be a mosaic due to somatic mutation. It is estimated that the frequency of crossing-over is as likely to lie below 5 % as above it.

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The Penetration into the Sea of Light of various Wave-lengths as Measured by Emission or Rectifier Photo-electric Cells

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This subject has engaged our attention since 1924, and papers dealing with it have been published already. The object of the present communication is to describe our latest methods of work with range increased one hundredfold, to discuss some sources of error and the precautions taken to minimize them, and to present further results, comprising eight spectral regions. The latter relate to a few series made with emission cells since the publication of our last communication (Atkins and Poole 1933) and other series carried out with rectifier cells and a modified method of measurement.

THE PHOTO-ELECTRIC CELLS

(*a*) *Emission Cells*

These were vacuum thin film caesium cells, which were used both under water and on deck with colour filters and a gas-filled sensitized potassium cell which was used for work under water at an anode potential of 12 V, a value below that at which an increase in sensitivity occurs owing to ionization by collision; it thus behaved like a vacuum cell as regards the rectilinear character of its light/current relation. The corresponding measurements on deck were made with a vacuum potassium cell.

(*b*) *Rectifier Cells*

All our later work has been done with the selenium cells of the Weston Electrical Instrument Co. These are sensitive throughout the spectrum with a maximum at about 595 mμ. Further details of both emission and rectifier cells may be found in our previous papers (Atkins and Poole 1933, 1936; Poole and Atkins 1935). The current from rectifier cells has to be corrected for curvature of the light/current relation.

Some of the more recent cells (without central blind spot) show large curvatures which may be intermediate between the linear and parabolic formulae, even with low resistances. The only course is to find (by bench tests or by comparison with a cell of known curvature) the curvature for each cell and for each resistance used and tabulate or plot the requisite correction factors. Where the Campbell-Freeth circuit is used, only the zero-resistance curvature is required for each cell; for this the factor k in the parabolic formula is only 0·0023 for the deck photometer.

COLOUR FILTERS

With the C.M.V. 6 emission cells the filters were the Corning glasses as previously used, with Schott R.G. 5 for dark red, and with the potassium cell Chance's red-purple ultra-violet transmitting glass. To minimize errors due to changes in the infra-red the C.M.V. 6 deck photometer had in addition a Corning heat-absorbing glass, light green in colour; this was omitted from the submarine photometer as the infra-red was, in any case, quickly cut out.

The rectifier cells were at first used (Poole and Atkins 1934*a*) with Chance's deep blue No. 7 which shows no appreciable red transmission, with Chance's No. 4, orange, Corning green and Schott R.G. 1, red. This set was given up in favour of a more extensive series of Schott filters of 2 mm. thickness. The reasons for selecting these filters are given in two recent papers (Poole and Atkins 1936; Atkins, Poole and Stanbury 1937), and their transmissions are shown in Schott's catalogue 4892E. The measurements made with the filters chosen, mounted as heretofore under opal flashed glass (opal), cover the spectrum from ultra-violet to infra-red. We have also introduced additional combinations so as to eliminate, as far as possible, results based upon the differences between measurements obtained with filters having different cut-offs.

Filters transmitting ultra-violet, but opaque to most of the visible spectrum, usually show also considerable deep red and infra-red transmission; with thin-film caesium emission or selenium rectifier cells the red must be eliminated by the use of an additional filter.

MEASUREMENT OF THE PHOTO-ELECTRIC CURRENT

For emission cells the potentiometer-amplifier-telephone null-method was used exactly as described in our previous communication. For rectifier cells we at first merely added a low resistance plug box to the outfit so that the difference could be measured across 10, 100, or 1000 ohms according as a single plug is inserted (Poole and Atkins 1934*a*). This aids rapidity of work. Spare terminals and a spare plug-hole permit the use of 10,000 ohms or multiples up to 100,000 ohms, as already fitted in the potentiometer-telephone apparatus. Curvature and temperature errors are both least with 10 ohms and nothing was to be gained by going beyond 10,000 ohms as the voltage to be measured was only slightly increased and the greater resistance resulted in a less easily obtainable potentiometer balance; but a good balance was always possible save under exceptionally damp conditions when the amplifier did not function so satisfactorily. As, however, the use of high resistance in series with the cell is most objectionable for several reasons, we had recourse to the Campbell-Freeth (1934) circuit, applied in a slightly modified form (Poole and Atkins 1934*b*). The method consists in the measurement of the current required to balance that generated by the cell and reduce the potential difference across the terminals of the latter to zero; it is thus equivalent to the use of a galvanometer of zero effective resistance, the only resistance in the circuit being that of the leads, which may be a few ohms. The modification lies in the deduction of the current from the setting of the potentiometer, from which it is derived, and the resistance through which it flows, instead of its direct measurement, thus eliminating the use of a galvanometer. The errors possible as a consequence of this use of the potentiometer have been considered, and have been shown to be unimportant when the resistance through which the balancing current flows is 100 ohms or more, provided a correction is introduced for the fact that the potentiometer system itself is now out of balance. When $R = 100$ ohms this correction never amounts to 10% with our 40 ohm potentiometer, and does not exceed 1% with $R = 1000$. With $R = 10{,}000$ or over it is negligible. A graph was provided from which it may be read off. Since the effective resistance of the measuring circuit remains zero, whatever the actual resistance through which the balancing current flows, it follows that the same calibration curve—and that the one with least curvature—holds for each cell for all resistances. Furthermore, temperature effects are a minimum, and one can work so that each scale division corresponds to 10^{-9} amp. By altering one resistance plug and by changing to the × 1 potentiometer setting full sunlight, over 100,000 lux may be measured. This degree of sensitivity (10^{-9} amp./scale division) is readily obtainable at sea on moderately dry days, but the apparatus is quite workable at 10^{-8} amp./scale division when housed in our trawler's small deck cabin throughout a day of almost incessant rain.

MARINE EQUIPMENT

The air photometer was mounted on gimbals and was placed on the deck-house roof in a position as free as possible from all shading.

The submarine photometers containing potassium and thin film caesium cells were those previously described and figured (Poole and Atkins 1926). For the rectifier cells a much smaller and lighter photometer was used.

No shutter is required with rectifier cells, as in the absence of external high potentials electrical leaks ("dark currents") do not occur. The absence of the strings needed to open and close the shutter used with emission cells is a further advantage in the use of rectifier cells; time is saved and risk of the cords becoming foul is abolished. More important still is the fact that it is possible to work in wet weather.

The submarine photometer is lowered into the sea by a cable passing through a pulley attached to a spar projecting over the stern so that the rolling of the ship does not affect the depth of immersion. Pitching, however, limits the length of the spar that can be used. Our practice has been to have the point of suspension slightly over 2 m. clear of the end of the ship's counter. Even in this condition the vertical motion may be considerable, say 1–2 m. in bad weather.

Precise details of the submarine photometer and its handling so as to avoid positional sources of error will be found in a forthcoming *Report of the International Committee for the Exploration of the Sea,* to which the authors have contributed.

MEASUREMENT OF THE SURFACE LIGHT

The emission and rectifier cells used were standardized as already described (Poole and Atkins 1935); recently the question of the agreement of the various photo-electric and visual scales has been considered more fully (Atkins and Poole 1936). Most fortunately it turned out that the earlier arc standardizations and the later ones in artificial mean noon sunlight were in satisfactory agreement; both are close to the visual under conditions of bright mixed daylight.

It is desirable to be able to give the results of the measurements in absolute energy units instead of in units which are strictly applicable only to visual observations. This has been done by Clarke (1933) using blue sensitive cells, and we have recently (Poole and Atkins 1936) standardized combinations of cells and filters to give the energy in microwatts per square centimetre per microampere of the photo-electric current. One can thus obtain the energy available for photosynthesis in certain broad spectral bands at a variety of depths.

Usually, however, we record the results of submarine measurements as percentages of the light in air. For this purpose the scale employed is immaterial, provided one bears in mind that only when dealing with a narrow band of wave-lengths can one obtain a uniform absorption coefficient in a selectively absorbing medium. As daylight is never constant, and often varies with great rapidity, the method of working was to make a rapid series of alternating readings of the two photometers, at each depth. Under good conditions six to eight single measurements could be made in 1 min. The cells and filters were as similar as possible. The readings of the two photometers were compared side by side in air, the submarine photometer having sea water in the spaces below the opal and between the stout plate glass and the colour filter. The under-water readings have throughout been multiplied by the factor 1·09 to correct for certain rather complex internal and external reflexion losses, for diffuse light, as previously discussed in detail (Atkins and Poole 1933).

Shading due to the Ship

A certain amount of shading is inevitable unless one lowers tubes with a photo-sensitive liquid suspended from a drifting spar as we did on a former (Atkins and Poole 1930) occasion, or unless one erects a floating support adequate for lowering the photometer as was done by Powell and Clarke (1936). Shading of direct sunlight was always prevented by keeping the stern towards the sun, so the error increases with the proportion of diffuse light. It has been shown by Poole (1936) that in diffuse light the error is a maximum, 10 %, at 5 m., which falls to 6 % at 10 m., and to 1 % at 40 m.

In bright sunlight, as only the diffuse light is affected by shading, the error would only be about one-third to one-sixth as great. We have not attempted to apply this correction to any of our measurements, as it is both uncertain and small in proportion to other possible errors.

Errors due to the Drift of the Ship

It is best to let the ship lie broadside to the wind with her stern in the direction nearest to the azimuth of the sun. In strong winds the drift will be rapid and the following errors (Poole 1936) will arise: (*a*) Deviation of the suspending cable from the vertical, which causes over-estimation of the depth. (*b*) The deflexion of the photometer window from the horizontal; provided the electric cable is attached to the top of the bridle this should not be serious. (*c*) Horizontal variation in the opacity of the water; if between two measurements at different depths the ship drifts into water of different average opacity, it is evident that the vertical extinction coefficient found from the ratio of the two sets of readings will be erroneous. It is well to observe the deviation of the cable from the vertical from time to time, but it is not easy to estimate the resulting error in the depth. If the supporting wire offered no resistance it would be straight, and the actual depth d would be $d' \cos \theta$, where d' is the apparent depth as read on the drum and θ is the angle with the vertical. On the other hand, were the supporting wire to cause the whole drag, the curve would be nearly the arc of a circle, the wire being vertical at the lower end, so that d would equal $d' \sin \theta/\theta$, where θ is in radians. From such considerations it was shown, taking $\frac{1}{3}d'\theta^2$ as a suitable approximation, that the depth error amounts to 1 % for an inclination of 10°, 4 % for 20°, and so on. Thus with $\theta = 20°$ the error at 50 m. would amount to 2 m.

Accuracy obtainable in Submarine Measurements

This is chiefly limited by the weather. On a calm cloudless day the surface light does not alter rapidly, the shading error due to the ship is small, and the depth of immersion constant to within about 0·1 m. Under such circumstances an accuracy of 1 % should be attained for each reading.

In bad weather with a strong wind and clouded or broken sky the light is very variable. Nevertheless by taking a long series and averaging, or by selecting a group of fairly concordant readings from such a series, no serious error is likely to arise in the measurement of the illumination in air at any rate. Pitching and drift of the ship may, however, render the depth of immersion variable and uncertain. Measurements within a few metres of the surface are particularly difficult in rough weather, so that it is usually wise to start at 5 m.

Since it is only in exceptionally clear water that blue or green light decreases by less than 10 % per metre, while both longer and shorter wavelengths are extinguished much more rapidly, it would seem that there is little advantage in measuring the light to much within 10 %, in conditions under which we may have an error of a metre in our depth as shown on the drum. In practice, however, it is only when the current has become extremely small that the readings could be anything like 10 % out for the actual mean depth.

In view, however, of the enormous range of illumination covered by most series of under-water measurements, an error even so large as 10 % is rarely of great importance. Thus it is not likely to matter very much whether, on a rough day, a certain percentage of the surface light is attained at 30 or at 31 m.

Opacity of the Water

For this we formerly used the term "vertical absorption coefficient", which we have denoted by μ_v. It is defined as being equal to

$$2{\cdot}30(\log_{10}p_1 - \log_{10}p_2)/d,$$

where p_1 and p_2 are the percentage illuminations at two points differing in depth by d metres. Reference may be made to our previous discussion (Atkins and Poole 1933) of this quantity. The term "extinction coefficient" is preferable as pointed out by Pettersson, for it describes a loss which is occasioned by both true absorption and by scattering.

Submarine Measurements

The two photometers were compared side by side on the deck-house roof or had been compared similarly on land. When calm enough a comparison was also made with the sea photometer swinging freely just above the water surface; the lead weight suspended below the rectifier cell photometer served to damp the oscillation since it dipped into the sea.

Series 82 obtained on 5 October 1932 with the C.M.V. 6 emission cell, off the Plymouth Breakwater Lighthouse, affords interesting measurements for relatively turbid shallow water, the depth being about 13 m. The light without any filter is reduced to 1 % at 10 m., and red light is down to 1 % at 4½ m. The sea was glassy and the sun shone through very thin white cloud; under these conditions, with a calm sea and the sun on the photometer, the illumination just above the water was 98·5 % of that on the deck-house roof, which shows that the shading by the ship was then negligible. The figures shown in Table III are based upon these results.

Series 84, 85 and 86 were obtained at International Hydrographic Station E 1 (about 20 miles out from the breakwater and about 10 miles south-west of the Eddystone Lighthouse). Potassium cells were used. In No. 84 Chance's ultra-violet transmitting red-purple glass was in position, μ_v was 0·196, 1–10 m. and 0·191, 10–20 m.; in No. 85 the cell had the opal only, and μ_v was 0·109, 1–10 m. and 0·101, 10–20 m. In No. 86 the photometer was suspended inverted, use being made of the lower shackle-holes as shown in fig. 1 of Atkins and Poole (1931). With the photometer suspended window downwards, about 3 m. above the water, the light reflected from the sea and travelling upwards was 6·45 % of that travelling downwards. At 5, 10 and 15 m. the ratio of the upwards to the downwards travelling light was 2·1, 2·0 and 1·2 %, respectively. The surface reflexion has recently been studied in detail by Powell and Clarke (1936), who also measured the light travelling upwards. Our results are quite in accord with theirs for similar water.

The results of Series W. 1, the first with the Weston selenium cell, have already been published (Poole and Atkins 1934*a*). These were obtained by the use of high resistances to measure the smaller currents with the ordinary circuit. On account of the large curvature corrections in certain cases we have omitted from this paper other determinations, made by this method, since they are of a lesser degree of accuracy than those now presented. The possibility of large curvature in some measurements of each series led to our changing over to the modified Campbell-Freeth circuit.

Series W. 8, W. 9 and W. 10 were obtained about 1½–2 miles east of the Eddystone, where much biological work has been carried out in regard to the vertical distribution of the zooplankton and otherwise.

In series W. 10, plotted in fig. 1, we used combinations of filters. This has obvious advantages, as it gives well-defined spectral regions directly. The disadvantage is that the light is so heavily cut down that one cannot follow all the combinations into deep water. The regions measured in these series were as follows: infra-red, R.G. 7, cut off at 760 mμ; deep red, R.G. 8, cut off at 700 mμ; dark red of series 82 and earlier, R.G. 5, cut off at 675 mμ; R.G. 1, at 600 mμ; O.G. 2, at 555 mμ; Corning grass green, 480–580 mμ, maximum 525 mμ; O.G. 2 + Corning green, 555–580 mμ; G.G. 11, G.G. 7, G.G. 5, G.G. 3, G.G. 2, cut off respectively at 500, 475, 445, 430 and 360 mμ; B.G. 12, 330–480 mμ; Chance's ultra-violet red-purple, 360–420 and 680 mμ upwards; the ultra-violet filter was used with B.G. 12, to measure the violet and ultra-violet band. Another narrow band was obtained by combining G.G. 5 with B.G. 12 to give appreciable transmission between 455 and 480 mμ.

The vertical extinction coefficients for series W. 8 and W. 10, taken almost a year apart, are shown in Tables I and II. In 1936 the water was considerably clearer than in 1935, for all spectral regions; this conclusion is supported by the coefficients of series W. 9, obtained during continuous rain.

Transmission is best in the middle of the spectrum and decreases at the ends. The effect of cutting off at the blue end may be detected in series W. 9, with four G.G. filters, 11, 7, 3 and 2, which between 15–25 m. (at which depths the red has been eliminated), gave respectively 0·162, 0·164, 0·168 and 0·176. The determinations occupied 40 min. only and were made in the sequence G.G. 7, 2, 11, 3, so the differences cannot be attributed to a

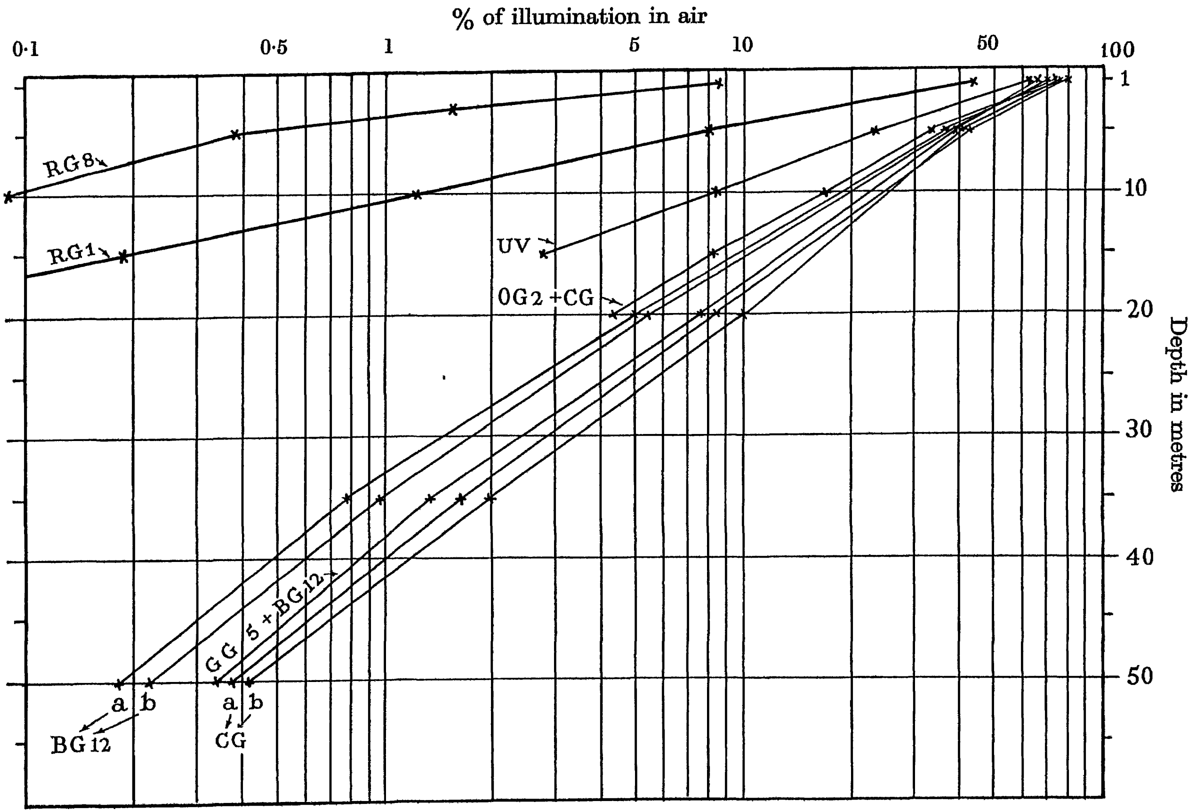

FIG. 1—Series W. 10, selenium cell. Drifting 1–3 miles east of Eddystone, 21 September 1936, depth about 55 m., wind light south-west, slight swell and small waves. Sky uniformly overcast with visibility only ½ mile at start, sun out with blue sky and clouds later. Secchi disk seen to 16 m. Filters lettered as before, with R.G. 8, deep red and B.G. 12+G.G. 5, blue green. B.G. 12 (*a*), determined at 11 a.m. G.M.T., and (*b*) at 2.30 p.m. Corning green (*a*) determined at 12.30 p.m. and (*b*) at 1.20 p.m.

change in the transparency of the water; they are scarcely significant, though in the correct order.

TABLE I—VALUES OF μ_v FOR VARIOUS SPECTRAL REGIONS

Series W. 8, selenium cell, 26 September 1935, 2 miles east of Eddystone, English Channel.

Long swell, wind light south-south-west. Sky hazy blue with sun through high cirrus. Secchi disk seen to 10 m. Depth 54 m.

Depth m.	No filter	R.G. 1	O.G. 2	Corning green
1– 5	0·217	0·435	—	—
5–10	—	0·412	0·257	—
10–15	0·157*	0·392	—	0·149*
15–20	—	0·277	0·203†	—
20–25	—	0·303	—	—
25–55	0·140‡	—	—	0·130§

* 5–25 m. † 10–20 m. ‡ 45–55 m. gave 0·155. § 25–45 m.

TABLE II—VALUES OF μ_v FOR VARIOUS SPECTRAL REGIONS

Series W. 10, 21 September 1936, ½ mile to east of Eddystone. Wind light, south-west. Uniform overcast sky, visibility only about ½ mile. Slight swell with ripples. Depth about 55 m. Secchi disk seen to 16 m.

Depth m.	λ mμ 700+	λ mμ 600+	λ mμ 555–580	λ mμ 480–580		λ mμ 455–480	λ mμ 330–480		λ mμ 360–420
1–2	0·855	—	—	—		—	—		—
2–3	0·755	—	—	—		—	—		—
3–5	0·703	—	—	—		—	—		—
5–7	0·725	—	—	A*	B	—	A†	B	—
1–5	—	0·425	0·216	0·156	0·145	0·116	0·173	0·146	0·260
5–10	—	0·373	0·138	—	—	—	—	—	0·202
10–15	—	0·379	0·143	0·095‡	0·109‡	0·112‡	0·133‡	0·133‡	0·222
15–20	—	0·284	0·131	—	—	—	—	—	—
20–35	—	—	—	0·107	0·108	0·114	0·123	0·114	—
35–50	—	—	—	0·104	0·099	0·093	0·098	0·099	—

* A set, at 12.30 p.m.; B set at 1.20 p.m.
† A set, at 11 a.m.; B set at 2.30 p.m.
‡ 5–20 m.

Apart from this alteration in transmission, due to changes in spectral composition, there does seem to be good evidence in series W. 10, that the deeper water from 35–50 m. is clearer than the water nearer the surface. We and others have had evidence for this before, and Pettersson's (1935) more direct method of measuring the transmission has given absolute proof of the existence of such differences in transmission existing in vertical water columns. In series W. 10 the measurements in the green and blue were repeated later in the day, and fairly concordant results were obtained showing that the water had changed but little during the work. The blue, the green and the band around 470 mμ give closely the same transmission in the clearest deep water, values slightly under 0·1 being shown. Even lower values have previously been found with the potassium cell at Station E1, μ_v being about 0·08 in the upper water of series 28, 29 and 41 (Poole and Atkins 1929). In series W. 10 the greatest amount of light at 50 m. was found with the green filter, namely 0·47 and 0·37 %, followed by 0·34 for the 470 mμ band, and 0·22 and 0·18 for the blue-violet.

The heavy absorption of the infra-red and the far red is noteworthy in view of the large amount of the total energy of the spectrum which lies in these regions. Using the R.G. 7 filter, which may be taken as cutting off at about 760 mμ, it was impossible to obtain steady readings as small waves had so much effect; values of from 2·4–9·3 % were, however, obtained for 1 m. depth, the latter figure giving μ_v 2·25. No appreciable amount of infra-red will, accordingly, reach 2 m.

The selective action of the water of series W. 10 may be appreciated by inspection of Table III which shows the depths at which the light passing the various filters, or of given wave-length, is reduced to 10, 1 and 0·1 %, as compared with similar values for series 82, near the Plymouth Breakwater. In the more turbid water of the latter, the selective action is much reduced. Thus near the Eddystone 0·1 % is reached for red light, R.G. 1, at 16·4 m., and for blue at 57 m., whereas inshore the depths are, respectively, 7·5 and 9·9 m.

Table III—Depths in Metres at which Light of given Wave-length*, in mμ, is reduced to 10, 1 and 0·1 % at the Eddystone, Upper Set, and at Plymouth Breakwater, Lower Set

λ mμ ...	700+	675+	600+	550–580	480–580	455–480	330–480	330–420	330+
λ max. ...	—	—	—	—	530	470	450	400	—
10 %	0·8	—	4·4	13·6	20	17·2	15	9·1	—
1 %	3·6	—	10·5	31·0	41	38	34	14·8	—
0·1 %	9·5	—	16·4	49·5	64†	53	57†	29·5	—
10 %	—	1·2	1·8	—	4·8	—	2·8	—	3·6
1 %	—	3·0	4·6	—	10·7	—	6·3	—	10·1
0·1 %	—	4·7	7·5	—	16·3	—	9·9	—	—

* Using filters R.G. 8, R.G. 5, R.G. 1, green+O.G. 2, green, B.G. 12+G.G. 5, B.G. 12, and ultra-violet+B.G. 12 and opal alone, respectively.

† Extrapolated, the depth is less.

The spectral energy distribution of the light at various depths is shown in fig. 2. The values for ultra-violet have been taken as at 400 mμ in view

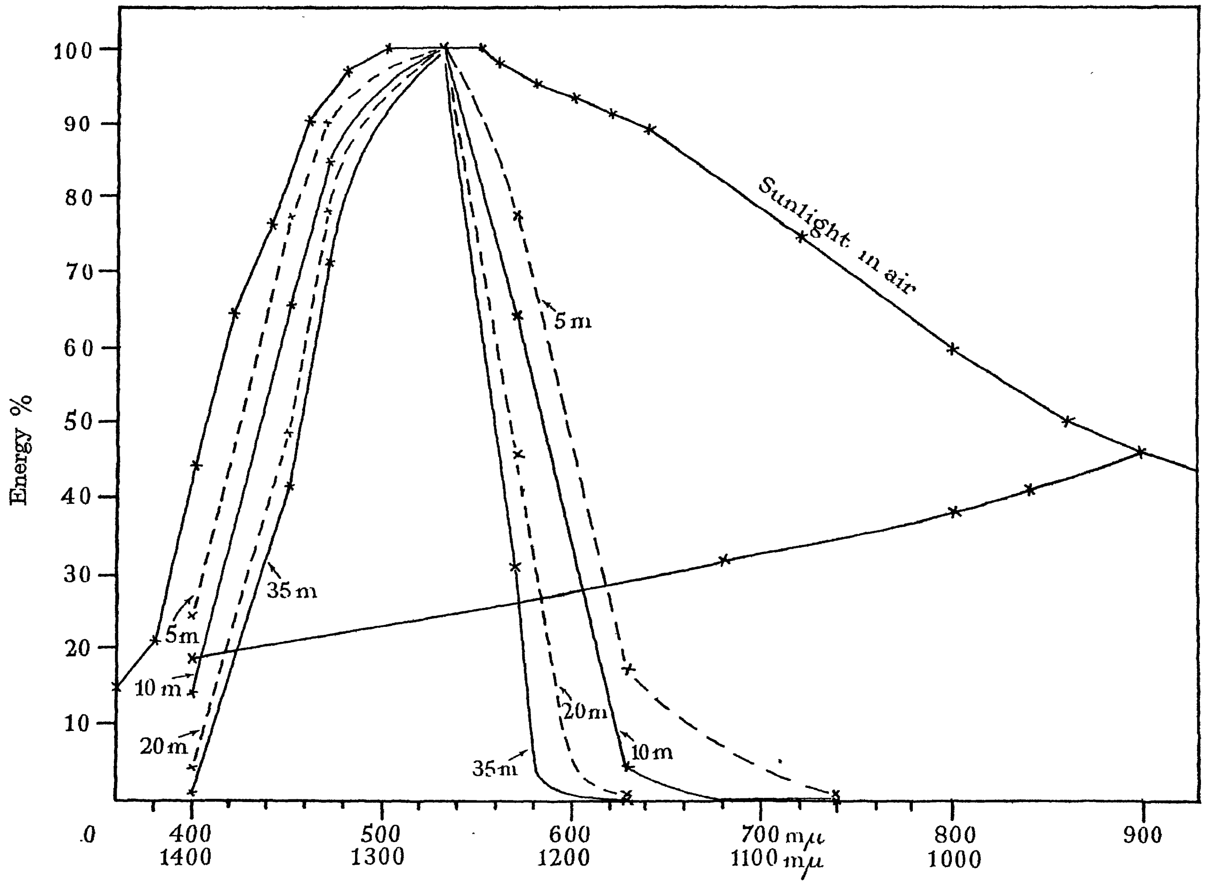

FIG. 2—The uppermost curve gives the spectral energy distribution of mean noon sunlight in air, continued to 1400 mμ to show the large amount of infra-red—which water cuts off. To economize space this curve is reversed at 900 mμ; the lower line of numerals refers to this portion. The other curves give the corresponding spectral energy distributions at 5, 10, 20 and 35 m. depth in the English Channel near the Eddystone.

of the rapid rise in intensity of the solar spectrum with increasing wave-length. The very heavy absorption of the infra-red is well brought out—it has practically vanished by 2 m. It may be seen that the spectrum at 35 m. consists mainly of green, with blue and a little violet and orange.

DISCUSSION OF RESULTS

The potassium cell results for blue light in series 84 obtained at E 1 show slightly greater penetration than the rectifier cell results with green filter in series W. 10, obtained 4 years later close to the Eddystone and hence nearer to the shore. The ultra-violet results for series 85 also give rather higher penetration in the offshore water. The measurements with inverted cell, indicating that the upward light is between 1 and 2 % of the downward, are in general agreement with previous measurements of our own, and with a much more complete set of measurements made by Utterback (1936) in the Pacific.

Series W. 8, W. 9 and W. 10 show a general similarity, the differences being mainly what one would expect from the different conditions under which they were obtained. Of these series the most interesting are probably the latest, plotted in fig. 1. The large difference in transmission between the spectral band 600–700 mμ, as measured by the selenium cell under R.G. 1, and the band 555–580 mμ, as measured with the combination of filters O.G. 2 and Corning green, is worth noting. The latter is an important region as it is so near the maximum of the solar energy spectrum.

The transmission for green light is not far from that commonly obtained in our earlier measurements with emission cells, while that for blue is intermediate between the rather widely scattered values indicated in figs. 2, 3 and 4 of our previous paper (Atkins and Poole 1933). It would seem that the transparency of water for blue light is more variable than that for green, as finely divided matter in suspension would have a greater effect on the shorter wave-lengths. Thus, in clear offshore water, and to an even greater extent in the oceanic water of the Sargasso Sea in which Clarke's deepest measurements were made, blue is the most penetrating, Clarke having obtained a value as low as 0·031 for the vertical extinction coefficient with a potassium emission cell. Figs. 2, 3 and 4, and Table II of his paper in the *Report of the International Council for the Exploration of the Sea*, 1936, show well the relative penetration of blue and green in different waters.

The very low extinction coefficient, 0·082, that Oster and Clarke (1934) have obtained for red light for the depth range 10–60 m. in the Sargasso

Sea would seem to suggest that wave-lengths close to the lower limit of transmission of the filter used are comparatively freely transmitted by ocean water, the relatively high extinction, from 0 to 10 m. about 0·36, being due to the absorption of the longer wave-lengths forming the greater part of the surface light transmitted by the filter, which was the Schott R.G. 1 with a $CuCl_2$ solution to cut off the longer wave-lengths. We have not so far found any confirmation of this effect with the R.G. 1 filter, nor has Utterback found such a high penetration for the red light transmitted by his filters. Clarke's values for the extinction coefficient of red light in inshore waters are quite in accord with Utterback's and our own.

Utterback, working with rectifier cells in the coastal and offshore waters of the Northern Pacific, has made very numerous measurements with filters transmitting comparatively narrow spectral bands. His results for offshore stations 20–100 miles off the land give average extinction coefficients for the range 10–70 m. as low as 0·068, the wave-length of maximum transmission for such waters ranging from 460 to 515 mμ. This Pacific water is thus more than twice as opaque as the clearest water found by Clarke in the Sargasso Sea, but considerably more transparent than the clearest water that we have so far encountered in the English Channel.

On the other hand, numerous measurements of inshore waters by Utterback, by Clarke, and by Pettersson in the Baltic and off the Swedish coast, give opacities in excess of those generally to be found a few miles off Plymouth.

There is thus, except for the high transmission of red found by Clarke in ocean waters, no disagreement between the results of different workers, nor is there as yet any further evidence of differences in opacity in different parts of the world apart from those caused by such obvious factors as distance from land.

We desire to express our thanks to the Marine Biological Association, Plymouth, for the use of the S.S. *Salpa*, from which the measurements were made, and to Capt. V. Lord and the crew for their skilful assistance with the gear, often under trying conditions. Our thanks are also due to the M.B.A. and the Government Grant of the Royal Society for defraying the cost of the apparatus and to the Royal Dublin Society for laboratory facilities in the standardization of the photo-electric cells.

SUMMARY

Further measurements of submarine daylight, using emission cells, gave for the vertical extinction coefficient 20 miles from land, $\mu_v = 0{\cdot}11$ for blue

and 0·19 for near ultra-violet. The light travelling upwards was about 2 % of that going downwards.

Measurements were made with Weston selenium rectifier cells, corrected for the curvature of the illumination/current relation, using a modified form of the Campbell-Freeth zero-resistance circuit. The method involves only a small change in the circuit of the potentiometer-amplifier-telephone null-method already described and avoids the use of a galvanometer. It permits of the measurement of full sunlight, which gives about five milliamperes, while weak light can still be measured with a sensitivity of 10^{-9} ampere per potentiometer scale division. The increase in range is thus one hundredfold. The rectifier cell can be used in wet weather since there are no electrical leaks ("dark currents").

Errors due to drift and the shadow of the ship have been considered. Colour filters were mounted under an opal flashed diffusing glass. Infra-red is eliminated in less than 2 m., giving μ_v about 2·2, with about 0·72 for deep red and 0·42–0·28 for red above 600 mμ, decreasing with depth as the average wave-length is altered. Red is reduced to 1 % at 10 m. For 550–580 mμ we find μ_v 0·22–0·13, for green 530 mμ, μ_v = 0·15–0·10 and for 450–480 mμ, μ_v = 0·11–0·10, with 0·17–0·10 for blue and 0·26–0·21 for ultra-violet. The deeper water, 35–50 m. was clearer than that near the surface in the English Channel 10 miles from land. Near the shore the difference between red and green is lessened, but blue is relatively more heavily absorbed and differs more from green.

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The Connexions of the Arcuate Nucleus of the Thalamus

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[Plates 4, 5]

As the result of detailed anatomical study, the ventral nucleus of the mammalian thalamus has been shown to be a composite structure, consisting of several nuclear elements which are to be distinguished by their cell and fibre architecture, and also by their fibre connexions. It has been demonstrated that medial fillet fibres (as well as ascending fibres from the spinal nucleus of the trigeminal nerve) terminate in the pars externa of the ventral nucleus (Vogt 1909; Clark 1936 *b*; Walker 1937), and that this element projects on to the gyrus postcentralis of the cortex. Brachium conjunctivum fibres, on the other hand, terminate in the rostral part of the ventral nucleus (extending forwards from approximately the plane of the anterior margin of the centre median nucleus) which, in turn, is connected by thalamo-cortical fibres with the motor cortex of the precentral gyrus (Clark and Boggon 1935). It appears, therefore, that, although the motor cortex certainly receives afferent fibres from the thalamus, these have not the same functional implications as the afferent fibres which reach the postcentral gyrus.

Of all the elements of the ventral nucleus of the thalamus, the best defined in higher mammals is the pars arcuata or arcuate nucleus (semilunar nucleus of Fleschig, nucleus *vb* of von Monakow and Friedemann, nucleus ventralis postero-medialis of other authors). The contour of this element can readily be determined by a naked-eye inspection of the freshly cut human or macaque brain. It is sharply circumscribed by a thin medullary capsule, it is darker in colour than the adjacent elements of the ventral nucleus, and it occupies a position in the postero-medial part of the thalamus immediately ventral to the centre median nucleus. The cells of the arcuate nucleus stain rather more deeply with methylene blue than those of the pars externa of the ventral nucleus, and they tend to be arranged in characteristic small clumps. In Weigert sections (fig. 5, Plate 4) the nucleus is not so richly permeated with fasciculi of medullated fibres, and these in general are distinguished by their fineness. The main part of the medial fillet can be seen to skirt its ventral and lateral margins to penetrate the pars externa of the ventral nucleus. Fine fibres run into the arcuate nucleus from the ventral medullary lamina of the thalamus and form within it a delicate interlacing network. The origin of these fibres is uncertain. Marchi studies following lesions in the spinal cord and medulla indicate that they do not arise from the spinothalamic tracts, the spinal nucleus of the fifth nerve, or the posterior column nuclei (Clark 1936 *b*). Ranson and Ingram (1932) reported that, in the cat, fibres of the brachium conjunctivum terminate in the "arcuate nucleus", and this conclusion seemed to be in accord with the observation (Clark and Boggon 1935; Clark 1936 *a*) that, in the monkey, the arcuate nucleus probably projects entirely on to the precentral gyrus. However, as has been previously pointed out, the "arcuate nucleus" as defined in the cat's thalamus is not wholly homologous with the arcuate nucleus of the macaque (or human) thalamus, and Marchi studies show no evidence that the thalamic fibres of the brachium conjunctivum terminate in the arcuate nucleus of the monkey's brain. Since, also, it has been shown that in the macaque brain the sensory cortex of the postcentral gyrus extends across the central sulcus to occupy the lower end of the precentral gyrus (Bucy 1935), it remains possible that the arcuate nucleus of the monkey is really related by ascending fibres to the granular area postcentralis (Brodmann's areas 1, 2 and 3) and not to the true motor cortex (Brodmann's area 4) as had earlier been assumed. The experiments recorded in this paper allow of a precise definition of the cortical connexions of the arcuate nucleus.

MATERIAL AND METHODS

Macaque monkeys (*Macaca mulatta*) were used for the experiments. In each case a block of cortex was removed under nembutal anaesthesia with the aid of an endothermy apparatus. The animals were allowed to survive for about two months, after which they were killed by an overdose of anaesthetic. At death the brain was immediately removed and studied macroscopically. The lesion was measured and as accurately as possible reproduced diagrammatically. The area of cortex containing the lesion was embedded in paraffin, sectioned and stained with methylene blue. In this way, the extent of the damage to the cortex could be ascertained, with special reference to the different cortical areas which had been involved.

As soon as the cortical lesion had been studied macroscopically, a block was removed from the brain containing the whole extent of the thalamus. This was placed in 70 % alcohol for 1 or 2 days until sufficiently hardened, when it was cut into separate blocks 2–3 mm. in thickness which were embedded in paraffin. Complete serial sections were cut throughout the length of the thalamus in a coronal plane at 15μ, and were stained with Borell's methylene blue.

Abbreviations used in the figures

a	Arcuate sulcus.
an	Anterior nucleus.
arc	Arcuate nucleus (pars arcuata of ventral nucleus).
c	Central sulcus.
cm	Centre median nucleus.
dm	Dorso-medial nucleus.
la *lb*	Elements of lateral nucleus.
S	Sylvian sulcus.
sbm	Nucleus submedius.
sth	Subthalamic nucleus.
ve	Pars externa of ventral nucleus.

EXPERIMENTAL RESULTS

O.M. 140. Operated 24 April 1936, killed 5 June 1936.

In this animal, a small localized lesion was made in the lower end of the postcentral gyrus. At death, surface inspection of the brain showed the lesion to be strictly limited anteriorly by the bottom of the central sulcus, and to extend up the posterior wall of this sulcus for its lower 8 mm. It involved the inferior extremity of the intraparietal sulcus, and, below, cut across the Sylvian sulcus to involve to a slight extent the adjacent part of the superior temporal gyrus. Here, also, it involved the surface of the caudal half of the insular cortex. The lesion measured 17 mm. in vertical diameter, and 9 mm. in maximum antero-posterior diameter. Sections cut sagittally through the middle of the lesion showed that the motor cortex (area giganto-pyramidalis) was not involved at all (fig. 1 *a*, *b*).

The Thalamus. The most caudal sections of the thalamus show that the cyto-architecture of the pulvinar is quite normal. There is a zone of complete cell atrophy in the lateral half of the rostral portion of the medial geniculate body, consequent on the involvement by the cortical lesion of part of the auditory area of the superior temporal gyrus.

At the level of the caudal extremity of the centre median nucleus an area of gliosis appears in the medial border of the ventral nucleus (pars externa) near its ventral end. As the sections are traced forwards, this is soon replaced by a sharply circumscribed patch of total cell atrophy in the medial half of the lower end of the ventral nucleus—on a level with the lateral margin of the centre median nucleus. This patch enlarges somewhat and becomes more distinct at the level of the middle of the centre median nucleus. Here it occupies the medial border of the pars externa of the ventral nucleus. In the lateral half of the pars externa there is quite distinct gliosis, but no evident cell atrophy. As the sections are followed forwards from the level of the middle of the centre median nucleus, the

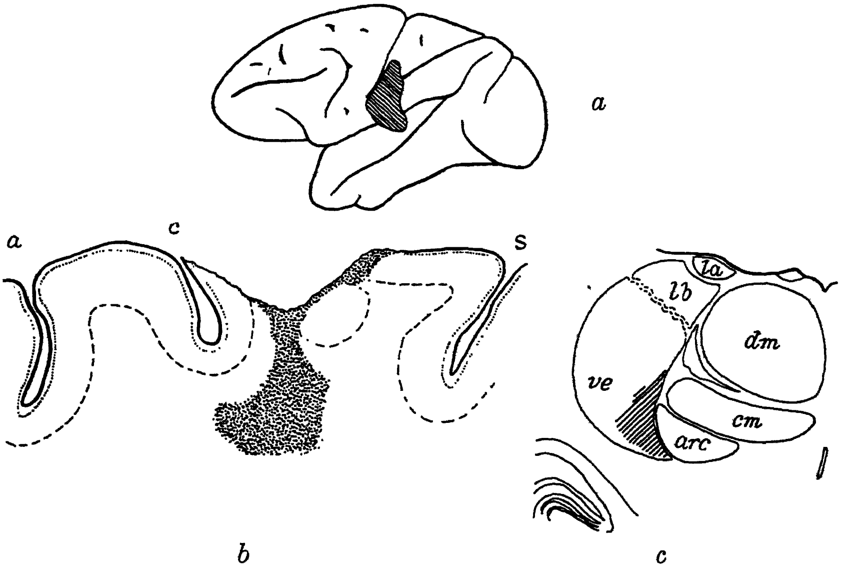

FIG. 1—*a*, the cortical lesion in O.M. 140, × ⅗. *b*, longitudinal section through the middle of the lesion, × 4. *c*, transverse section through the middle of the thalamus showing the extent of the cellular atrophy (hatching) in the pars externa of the ventral nucleus.

area of cell atrophy diminishes rather rapidly. It becomes limited to the medio-ventral extremity of the pars externa, while the pars arcuata at its maximum development in transverse section shows a completely normal cyto-architecture (fig. 1 *c* and fig. 6, Plate 4). Finally, at the level of the rostral extremity of the lateral geniculate body and the middle of the dorso-medial nucleus, and near the rostral border of the centre median nucleus, the patch of cell degeneration shrinks to the extreme medio-ventral point of the pars externa of the ventral nucleus and disappears altogether.

O.M. 55. Operated 27 March 1936, killed 6 May 1936.

As seen in fig. 2 *a*, the main lesion in this case involved the cortex immediately adjacent to the Sylvian sulcus, extending anteriorly into the lower extremity of the postcentral gyrus.

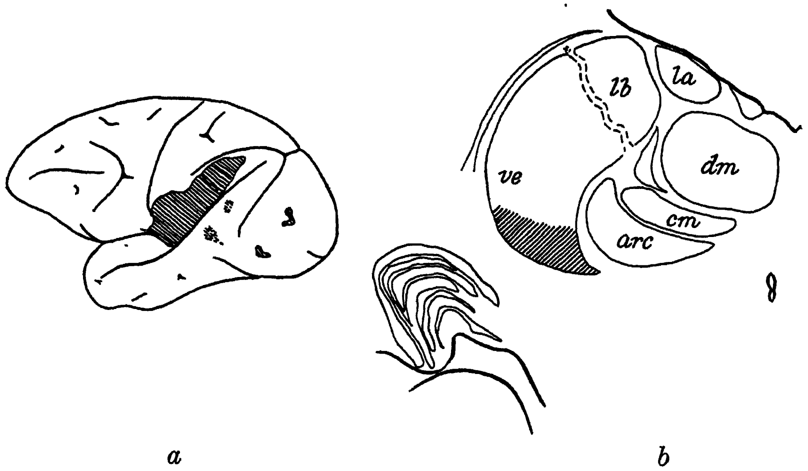

Fig. 2—*a*, cortical lesion in O.M. 55, ×⅗. *b*, transverse section through the middle of the thalamus showing the extent of the cellular atrophy in the pars externa of the ventral nucleus.

The Thalamus. Caudal sections of the thalamus show extensive cell atrophy in the medial geniculate body and the pulvinar. This degeneration is secondary to the involvement of the auditory cortex and the posterior Sylvian receptive area, and has been described in detail elsewhere. The injury to the lower end of the postcentral gyrus is related to a sharply defined patch of complete cell atrophy in the ventral extremity of the pars externa of the ventral nucleus (fig. 2 *b* and fig. 7, Plate 5). This area of atrophy extends as far rostrally as the level of the anterior margin of the centre median nucleus. The arcuate nucleus is entirely normal in its cytoarchitecture, except only for a suggestion of partial atrophy at the middle of its lateral border in more caudal planes. This, however, is so slight as to be quite indefinite.

O.M. 113. Operated 19 December 1935, killed 19 February 1936.

In this experiment (which has been reported elsewhere) the entire parietal lobe was extirpated on one side. From the point of view of the present study it is to be noted that the lesion involved the whole length of the exposed part of the postcentral gyrus, reaching down to the level of the inferior extremity of the central sulcus. The posterior wall of the sulcus was not involved.

Sections of the thalamus show that, while there is extensive cell atrophy in the pulvinar and in the pars externa of the ventral nucleus, the arcuate nucleus is completely normal in its cyto-architecture.

O.M. 142. Operated 29 April 1936, killed 11 June 1936.

In this experiment, the intention was to produce a localized lesion in the inferior extremity of the precentral gyrus. However, inflammatory adhesions led to a destruction also of part of the cortex in the inferior extremity of the postcentral gyrus, in the middle third of the superior temporal gyrus, and in the most rostral part of areas 18 and 19 (close to the rostral convexity of the lunate sulcus). At death, the lesion measured 24 mm. in vertical diameter. The extent of the lesion as seen macroscopically is indicated in fig. 3 *a*. Microscopic sections through the lesion showed that the cortex over this region had been almost completely destroyed or had undergone degeneration. It should be noted that, rostrally, the lesion cuts across the arcuate sulcus and extends into the caudal part of the granular frontal cortex (areas 8 and 9).

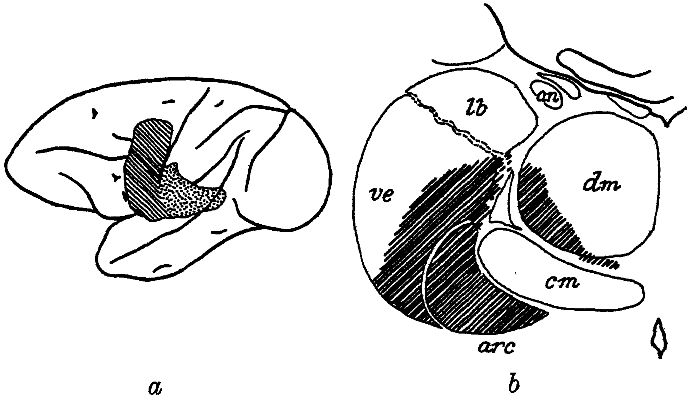

FIG. 3—*a*, cortical lesion in O.M. 142, × ⅗. *b*, transverse section through the middle of the thalamus showing the extent of the cellular atrophy in the pars externa of the ventral nucleus, the arcuate nucleus, and the dorso-medial nucleus.

The Thalamus. At the caudal extremity of the thalamus an area of gliosis and cell atrophy appears in the centre of the pulvinar. This rapidly becomes more distinct as the sections are traced forwards, and soon extends over the greater part of the medial half of the pulvinar from its dorsomedial angle down to the level of the corticotectal fasciculi which penetrate it. At the rostral end of the pulvinar (in sections through the caudal extremity of the habenular ganglion), the patch of atrophy is very distinct and clear-cut, occupying here the medial third of the pulvinar (element *p*β), while one or two very small and isolated patches are to be seen more laterally. Farther forwards, element *pd* of the pulvinar appears in the serial sections. This nucleus is also affected by considerable, but not complete, cell degeneration. The area of degeneration also involves the caudal extremity of the pars externa of the ventral nucleus. Elements *pa* and *pla* of the pulvinar are quite unaffected. The caudal end of the medial geniculate body is completely degenerated. At this level the area of cell atrophy in the pulvinar becomes rapidly restricted to a relatively small patch close to the middle of its medial border. A few sections farther forwards, the centre median nucleus appears and, as is always the case in cortical ablations, its cyto-architecture is perfectly normal.

In sections passing through the middle of the dorso-medial nucleus, there is a very distinct and clear-cut zone of atrophy at the ventrolateral and ventral borders of this element, the result of the involvement by the cortical lesion of the frontal areas (areas 8 and 9). At this level the area of atrophy in the ventral nucleus occupies the medial border of the pars externa and extends ventro-medially into the arcuate nucleus. Farther forwards, the zone of atrophy in the ventral nucleus becomes rather more extensive, but retains its sharp contour. The arcuate nucleus is completely degenerated (fig. 3 *b* and fig. 8, Plate 5). The lateral half of the pars externa remains unaffected. Beyond the level of the rostral margin of the centre median nucleus, the zone of degeneration in the dorso-medial nucleus soon disappears, while the atrophic area in the ventral nucleus also shrinks rapidly until it comes to occupy the ventral third of the ventral nucleus.

O.M. 149. Operated 17 September 1936, killed 18 November 1936.

In this experiment, a small lesion was made in the lower end of the motor cortex. The lesion was planned with the intention of limiting the injury to the motor cortex without involvement of that portion of the lower end of the area postcentralis which extends in front of the lower extremity of the central sulcus. In order to do this, reference was made to the diagrams of the extent of the motor cortex in a series of macaque monkeys given by Bucy (1935) in his paper on the comparative cyto-architectonics of the motor area in the Primate cortex. At death the lesion was found to measure 17 mm. in vertical diameter and 5 mm. anteroposteriorly. Its lower margin was 6 mm. above the inferior extremity of the central sulcus, and its anterior margin 2 mm. from the arcuate sulcus. Serial sections were cut transversely across the lower half of the lesion and stained with methylene blue. These show that the lesion was limited to the motor area proper (fig. 4 *a*, *b*).

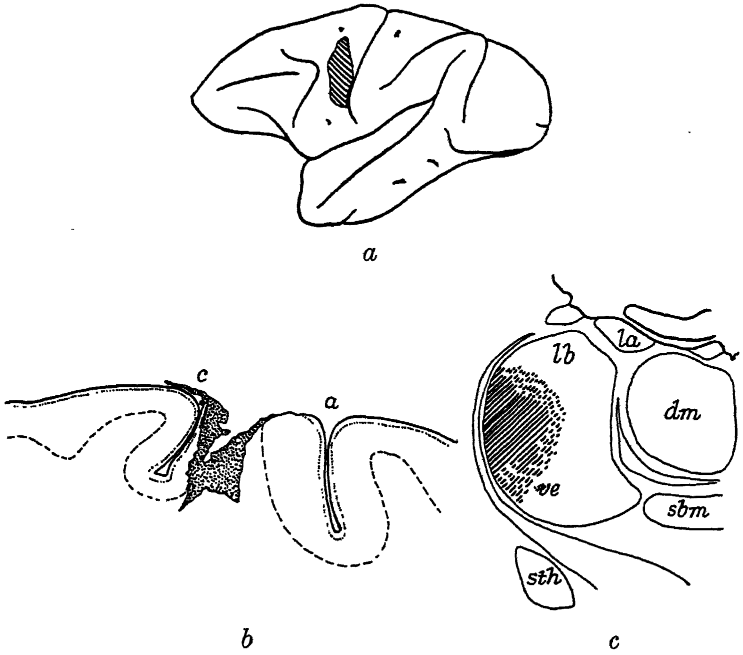

FIG. 4—*a*, the cortical lesion in O.M. 149, × ⅗. *b*, sagittal section through the middle of the lesion, × 4. *c*, transverse section through the middle of the thalamus showing the cellular atrophy in the rostral part of the ventral nucleus.

The Thalamus. Serial sections show no cell degeneration in the caudal half of the thalamus. The arcuate nucleus is quite normal. There is evidence of slight gliosis in the medial medullary lamina at the lateral border of the centre median nucleus, but this is not definite. At the transverse level of the rostral border of the centre median nucleus, an extensive area of almost complete cell atrophy with accompanying gliosis is present in the pars externa of the ventral nucleus, reaching dorsally as far as the lateral nucleus (fig. 4 *c*). This area extends forwards in the same relative position as far as the level of the anterior nucleus, becoming limited more and more to the lateral margin of the ventral nucleus. In sections at the level of the middle of the anterior nucleus, no further cell atrophy in the thalamus is to be seen.

O.M. 88 and *O.M.* 102.

The results of these experiments have been recorded elsewhere in connexion with a study of the temporal lobe (Clark 1936 *a*). In both cases, the cortical lesion involved to a slight extent the lower opercular lip of the precentral gyrus, well in advance of the lower extremity of the central sulcus. In both cases, also, the lesions were followed by very clear-cut zones of complete cellular atrophy in the arcuate nucleus, at or near its medio-ventral end. The pars externa of the ventral nucleus was normal in its cyto-architecture.

DISCUSSION

The series of seven experiments recorded above leads to the conclusion that the portions of the granular area postcentralis which lie in front of and behind the lower end of the sulcus centralis in the macaque cortex receive afferent fibres from different thalamic elements. Both these parts of the area postcentralis lie in the sensory area of representation for the face as defined by Dusser de Barenne (1924) on the basis of his strychninization experiments, and therefore presumably receive impulses conveyed by the trigeminal system. The postcentral portion of the lower end of the sensory cortex is connected with the medio-ventral extremity of the pars externa of the ventral nucleus, while the arcuate nucleus projects on to the precentral portion. The medio-ventral extremity of the pars externa of the ventral nucleus has been shown to receive secondary fibres from the spinal nucleus of the fifth nerve in the monkey (Clark 1936 *b*), and Wallenberg's observations on the rabbit (1895 and 1900) appear to lead to the same conclusion. These ascending and cortical connexions of the pars externa show that by no means the whole of the trigeminal fillet terminates in the arcuate nucleus, as has been recently suggested by Walker (1937).

That a portion of the trigeminal fillet ends in the arcuate nucleus seems almost certain, though this has not yet been demonstrated by direct methods. The medial position of the nucleus in the thalamus suggests such a connexion, for the fibres of the trigeminal fillet are known to enter the thalamus at the medial end of the medial fillet and hence are brought into close topographical relation with the arcuate nucleus. In a series of experimental observations recently made on the termination of ascending tracts in the thalamus of the macaque monkey (Clark 1936 *b*), it was inferred that the nucleus probably receives secondary fibres from the medullary nucleus of the fifth nerve. However, attempts to place a localized lesion in this nucleus in order to decide this question by the Marchi technique were not successful. The fact that the nucleus projects on to the "face" area of the sensory cortex, however, makes it certain that it is the recipient of some of the secondary trigeminal fibres. The nature of the impulses which these fibres carry is suggested by an observation of Dusser de Barenne (1924). In an experiment on a macaque monkey (No. XVII), he applied a weak strychnine solution to the opercular lip at the lower end of the left precentral gyrus and outside the excitable motor area, that is to say, to cortex which is now known to be a part of the projection area of the arcuate nucleus. As a result of this, the animal was observed to open and shut the mouth frequently, protruding the tongue and licking its right upper lip and the right angle of its mouth. Dusser de Barenne regarded these movements as symptoms of spontaneous, paraesthetic sensory disturbances in the region of the tongue and jaws. In another experiment by the same observer (No. VIII), the strychnine was applied to the postcentral part of the lower end of the sensory cortex, and in this case, while no jaw and tongue movements are recorded, evidence of hyperaesthesia over the scalp and ear was found. Taken in conjunction with the present observations on the thalamic connexions of these regions of the cortex, these results lead to the inference that the arcuate nucleus receives trigeminal impulses mainly from the tongue and mouth region, while the sensory impulses from the rest of the trigeminal area are transmitted to the pars externa of the ventral nucleus. In this connexion, it may be noted that the projection area for the arcuate nucleus is immediately contiguous with the excitable motor cortex, stimulation of which gives rise to movements of the tongue and jaws.

The cost of the animals used in this research was met by a grant from the Royal Society, which I wish gratefully to acknowledge.

SUMMARY

Experiments are recorded which show that the arcuate nucleus of the thalamus projects on to an area of granular cortex occupying the lower end of the precentral gyrus, extending over the opercular lip which lies between the central sulcus behind and the arcuate sulcus in front. Above, the area reaches up to the inferior extremity of the motor cortex (area giganto-pyramidalis). Evidence is adduced showing that the arcuate nucleus is concerned with the cortical projection of impulses from the tongue, mouth and lip region.

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DESCRIPTION OF PLATES

Plate 4

FIG. 5—Transverse section through the middle of the thalamus of a macaque monkey, showing the position of the arcuate nucleus of the thalamus (*arc*). Other nuclei shown are the pars externa of the ventral nucleus (*ve*), elements *la* and *lb* of the lateral nucleus, the dorso-medial nucleus (*dm*), the centre median nucleus (*cm*), and the red nucleus (*r*). × 5.

FIG. 6—Transverse section through the thalamus of O.M. 140, showing the area of cell atrophy (*A*) in the pars externa of the ventral nucleus (*ve*). The cells of the arcuate nucleus (*arc*) and the centre median nucleus (*cm*) are normal. × 27.

Plate 5

FIG. 7—Transverse section through the thalamus of O.M. 55, showing the area of cell atrophy (*A*) in the medio-ventral extremity of the pars externa of the ventral nucleus (*ve*). × 21.

FIG. 8—Transverse section through the thalamus of O.M. 142, showing the area of cellular atrophy (*A*) in the pars externa of the ventral nucleus, and the complete cell degeneration in the arcuate nucleus (*arc*). × 21.

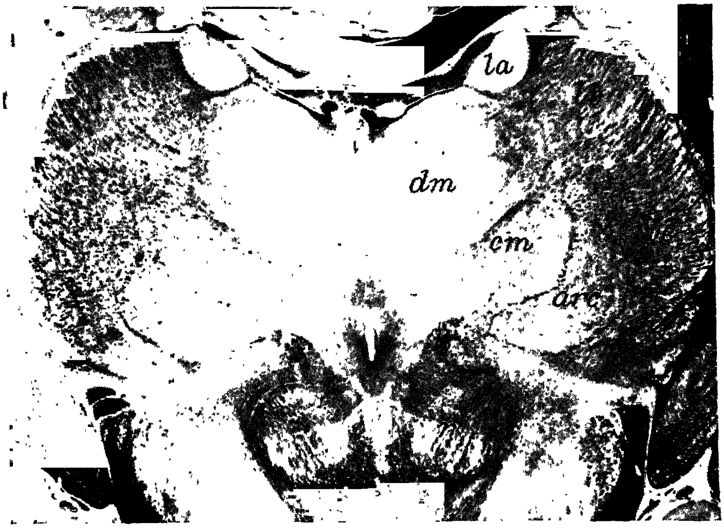

Fig. 5

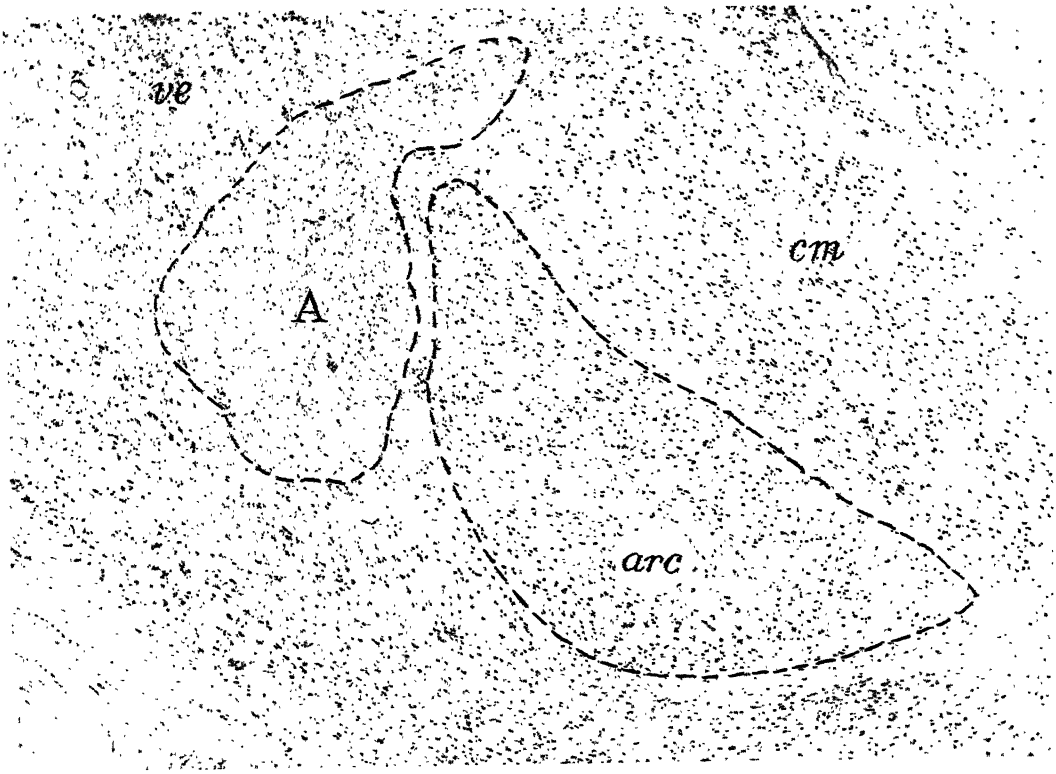

Fig. 6

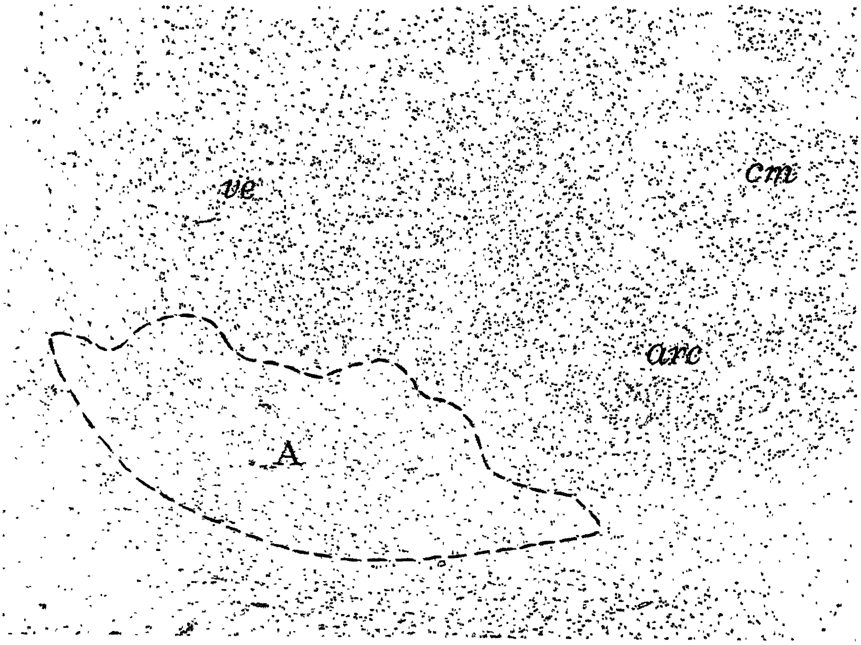

FIG. 7

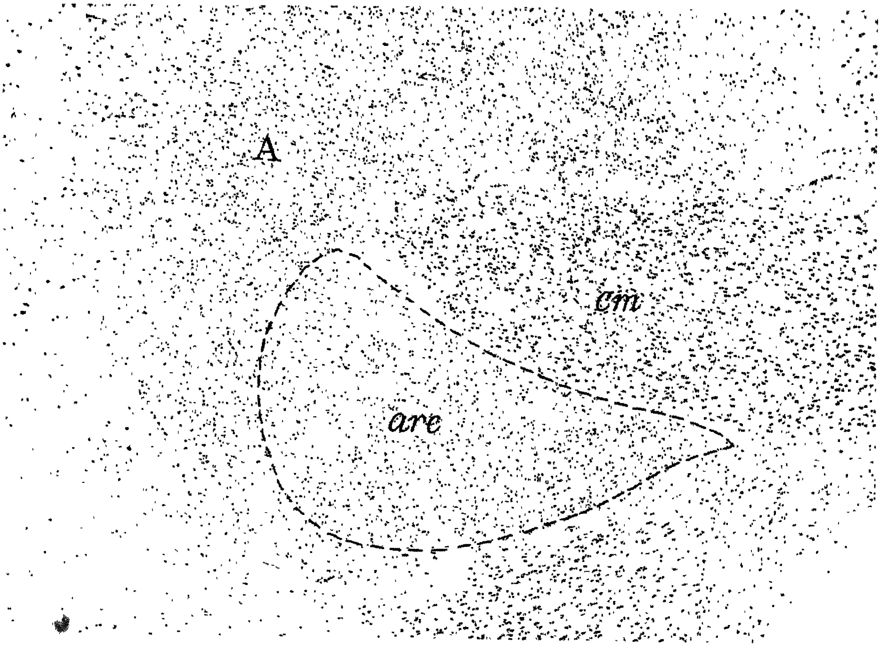

FIG. 8

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On the Response of the Human Ear to Audio-frequency Electrical Stimulation

BY C. S. HALLPIKE* AND H. HARTRIDGE, F.R.S.

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[Plate 6]

INTRODUCTION

In a short paper published in 1930, Jellinek and Scheiber stated that alternating currents from an audio-frequency generator, when passed through the head of a human observer, set up sensations of tone corresponding roughly in pitch to the frequency of the stimulus. Further investigations by Perwitzschky (1930), Fromm, Nylen, and Zottermann (1933), and by Gersuni and Volokhov (1934) have since confirmed this finding. In addition, the possibility that the tympanic membrane and ossicles are concerned in the production of the phenomenon has been excluded by Perwitzschky (1930) and by Gersuni and Volokhov (1934), who describe its persistence following the surgical removal of these structures. If the likelihood be accepted that excitation under these circumstances is brought about by direct action upon the terminal fibres of the cochlear nerve, then the bearing of the phenomenon upon prevailing views on audition lies clearly in the support which it provides for the telephone or frequency theory. There is nothing to suggest the probability that electric currents traversing the cochlea would be localized according to frequency to those areas which, in accordance with the resonance hypothesis, would be activated by physiologically applied sound waves. Hence, there is implied a simultaneous involvement of all the fibres of the cochlear nerve irrespective of their localization upon the basilar membrane, and the transmission by the nerve trunk of the stimulus frequency itself, as postulated upon the telephone theory. An alternative possibility, and one, moreover, which has been considered by all the investigators cited, is that the electrical changes, in a manner not understood, cause movements of those cochlear elements which are normally involved by sound waves. Upon such a basis the observed phenomenon would be clearly explicable upon the resonance hypothesis of Helmholtz.

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The experimental work to be described in this paper has as its objective the establishment of further experimental data bearing upon this "movement" hypothesis of excitation by audio-frequency electrical stimulation.

In this work a further application has firstly been made of the principles involved in the "phase reversal beat". As previously shown by Hartridge (1921), the sudden reversal in phase of a continuous musical tone causes in man the sensation of a beat. Objective evidence bearing upon the mechanism of this beat has further been obtained in a recent series of experiments (Hallpike, Hartridge, and Rawdon-Smith 1937) in which transient silent periods were demonstrated in the auditory tract potentials of the cat in response to sudden phase reversals in continuous musical tones. These two pieces of evidence taken together appeared to justify the view that the beat heard by the human subject has as its basis, at any rate partly, a transient silent period in the auditory tract responses. The explanation of the silent period is provided on the basis of the Helmholtz resonance theory of hearing as follows: Immediately following the phase reversal, the fibres of the basilar membrane do not immediately follow the reversal in phase, but due to their persistent after-swings have first to be brought to rest, and then to be restarted in the new phase. Owing to the mass of these elements, this arrest and recommencement must occupy a certain space of time, and during this time the responses of the auditory nerve will be below that produced by the unmodified tone. Under these circumstances, the beat would clearly bear a direct relationship to the phenomenon of flicker produced by transient interruptions in excitation in this and other sensory modes. If the intensity of any such flicker-producing stimulus be increased from a subliminal value, then the background is first perceived as an unbroken sensation. Flicker perception is only attained on further increasing the intensity. The amount of this increase varies inversely with the length of the flicker-producing interruption. In any given set of circumstances, a certain gradient in the fall of sensory activity is produced, and in the case of auditory flicker, it seems certain that an important part in determining the slope of such a gradient must be played by resonance in the structures initiating the sensation in question. In the experiments which have been performed, the phase reversal beat has been treated upon the lines of a flicker phenomenon, and a determination made of the intensity increase above threshold value which is required for perception of the beat.

The phase reversing stimulus was applied in the first place acoustically, that is to say, by means of a moving-coil loud-speaker. As already stated, the physical characteristics (in particular, their coefficient of damping) of the resonant elements of the internal ear involved mechanically in this mode of stimulation must be taken as playing an important part in determining the slope of the sensory gradient which, in turn, determines the intensity increase referred to. That is to say, greater or less degrees of damping would accentuate or decrease respectively the steepness of this gradient. Consequently, the degree of correspondence of such an intensity increase with its counterpart similarly determined in the case of electrical stimulation of the ear by a wave series of identical form would provide strong evidence bearing upon the hypothesis that these resonant elements are similarly involved by electrical excitation. Observations similar in type have also been made in the case of beats which result from transient interruptions in musical tones. Here also, the stimulus has been applied both acoustically and electrically.

TECHNICAL METHODS

The phase reversing photo-electric siren recently described (Hallpike, Hartridge, and Rawdon-Smith 1937) was used as a sound generator. It was also used, as will be described later, for generating the tones which were interrupted for known time intervals. The amplified output of the photocell of the siren was connected via a correctly matched decibel attenuator either to the power stage which fed the loud-speaker or, via a second attenuator, to a power stage of somewhat greater gain which fed the electrodes that were used for stimulating the observer's ear. One of these electrodes consisted of an enamelled copper wire, cemented along the inside of a celluloid aural speculum with the insulation removed from its free end. The other electrode consisted of a lead plate roughly 5 cm. square, which was placed in firm contact with the moistened skin of the observer's forearm. Over all, the moving-coil receiver which was employed for acoustic stimulation was firmly secured. A diagrammatic representation of the circuits is given in fig. 1. Visual and photographic control of the wave form of the electrical and acoustic stimulation employed was provided by means of the cathode ray oscillograph, microphone and moving-film camera equipment, which has been fully described elsewhere (Hallpike and others 1937).

EXPERIMENTAL PROCEDURE

The experimental procedure which was carried out by two observers (C. S. H. and H. H.) is described as follows: The observer's head was placed sideways on a firm rest and the upper internal auditory meatus was filled with warm normal saline solution. Electrical stimulation was carried out by means of the electrode arrangement already described and, for stimulation by means of sound, the loud-speaker was hung upon an elastic suspension in order to facilitate its application to the ear. By means of the two attenuators mentioned above it was possible to vary the electrical and acoustic energy applied to the ear independently and by known amounts.

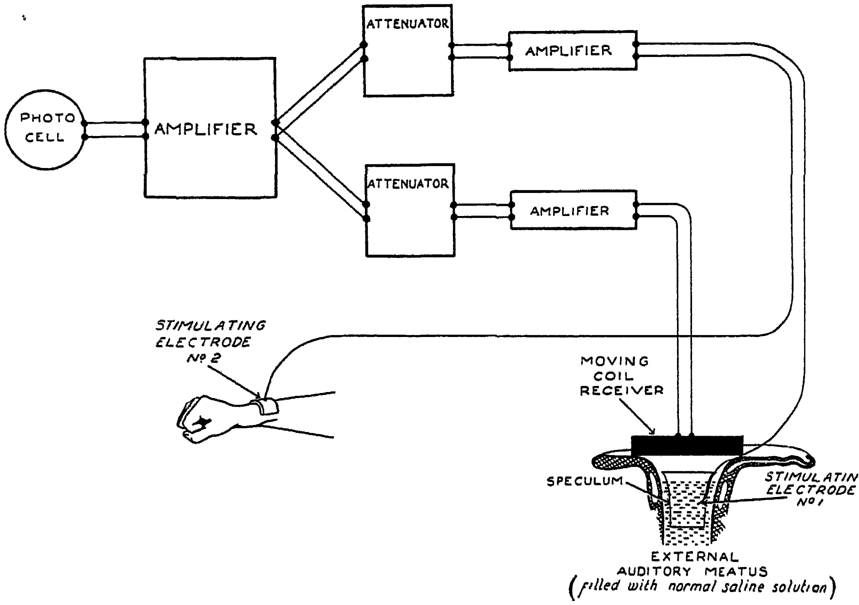


FIG. 1

It will be noted that the external auditory meatus of the stimulated ear was filled with saline solution for both acoustic and electrical stimulation. This was done in order to equalize for the two methods any possible effect that might be produced by the mass of the solution. The degree of hearing loss resulting from the fluid mass in the external auditory meatus was found to amount to some 30 db.

In order to avoid the possibility of contralateral stimulation at the relatively high intensity thus required, the meatus of the opposite ear was occluded by means of a plastic plug. This arrangement had the further advantage of effectively excluding extraneous sound.

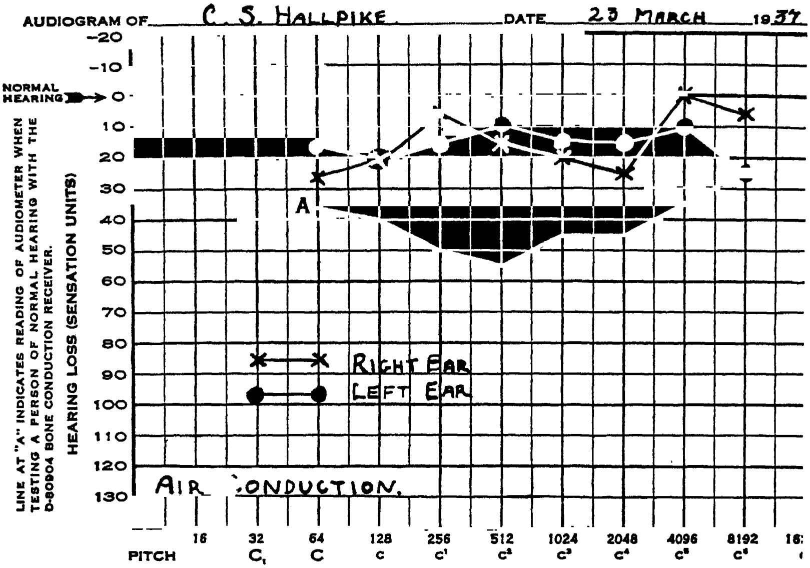
PRELIMINARY INVESTIGATIONS

By way of introduction to the experimental results to be described, reference may here be made to certain preliminary investigations which were found to be necessary:

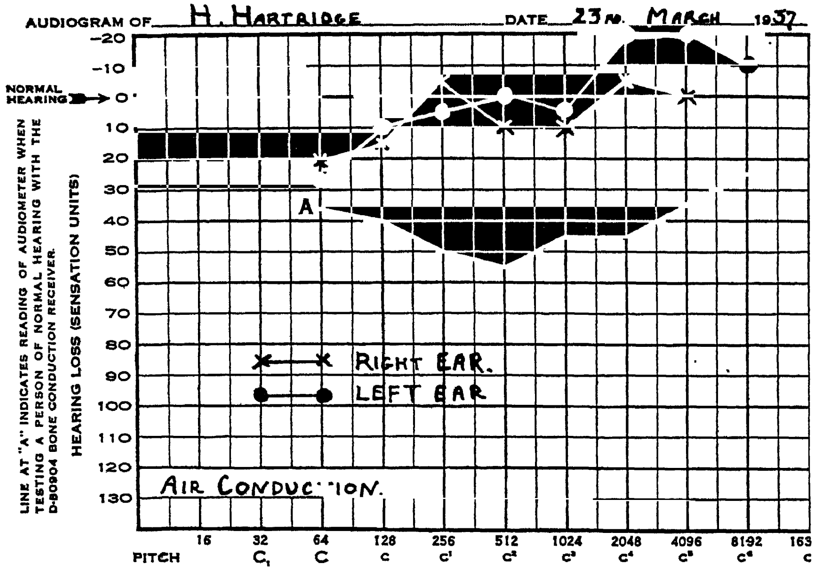
(*a*) *The hearing of the two observers concerned* (*H. H. and C. S. H.*). The audiograms are reproduced in fig. 2. These show normal hearing in the case of H. H. C. S. H., however, has a small but definite loss throughout the range tested. The right ear of each observer was employed in the present experiments.

(*b*) *The degree of correspondence in pitch in the sensations of tone evoked by electrical and acoustic stimulation employing a wave series of given form and frequency*—As originally described by Jellinek and Scheiber (1930) this correspondence in respect of pitch is by no means absolute. Gersuni and Volokhov (1936) and Barany (1937) have in particular stressed that at low frequencies of electrical stimulation upper partials may be marked and may in some cases dominate the fundamental. The matter was accordingly investigated in the case of the two observers concerned in the present experiments (C. S. H. and H. H.), and at the actual frequencies of stimulation employed, namely 512 and 1024~. In the case of both observers, both acoustic and electrical stimulation at 512 and 1024~ was found to give rise to sensations of tone corresponding exactly in pitch to those produced by tuning forks of these frequencies placed upon the skull. Strong beats were heard on slightly varying the frequency of the electrical or acoustic stimulus. Similar, but much weaker, beats were also heard on testing in a similar manner with tuning forks of frequency one octave higher than that of the acoustic or of the electrical stimulus. These findings were considered to provide satisfactory evidence that under the experimental conditions in use, there was in fact a precise correspondence in the pitch of the tonal sensations brought about by acoustic and electrical stimulation.

(*c*) *The relative sensitivity of the ear to fractional increases in acoustic and electrical energy at juxta-liminal intensity values*—It was found that a marked difference in this respect was to be observed in the reactions of the two observers presently concerned. This difference is made apparent by a comparison of the two graphs shown in figs. 3 and 4. From these it may be seen that in the case of one observer (H. H.) there was found in the neighbourhood of the threshold a close correspondence in the subjective responses to fractional increases of acoustic and electrical energy of given amount.



(*a*)



(*b*)

Fig. 2—Audiograms of H. H. and C. S. H. Western Electric Audiometer Type 2 B.E.

In the case of the other observer, however (C. S. H.), it may be seen that a relatively small fractional increase of electrical energy evoked a rise of sensation which was found to correspond to a considerably greater fractional increase of acoustic energy. This was particularly marked in the region immediately above the threshold. It may be noted that this inequality in the subjective response to fractional increases in intensity of electrical and acoustic stimulation is in accordance with results recently published by Arapova, Gersuni, and Volokhov (1937). These findings have an obvious bearing upon the mode of interpretation of the experimental results which are to be described, and indicate with some clearness the impracticability of making any comparisons of the central responses to electrical and acoustic stimuli in terms of their physical energy. For the purpose therefore of the experimental results to be described, it becomes necessary to make such comparisons solely upon the basis of psychological intensity, that is to say, by comparing the levels of loudness above threshold

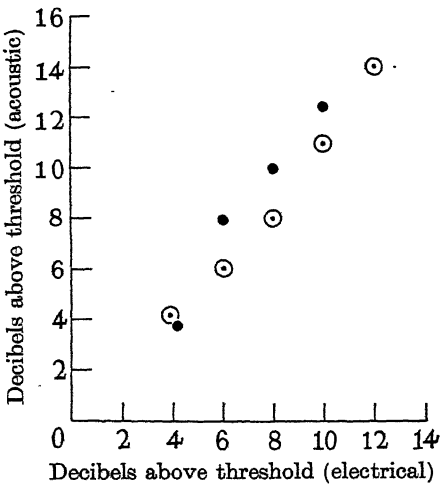


FIG. 3—Intensity difference (log I/I_0) sensitivity for acoustic and electrical stimulation. Observer H. H. 512 ●. 1024 ⊙.

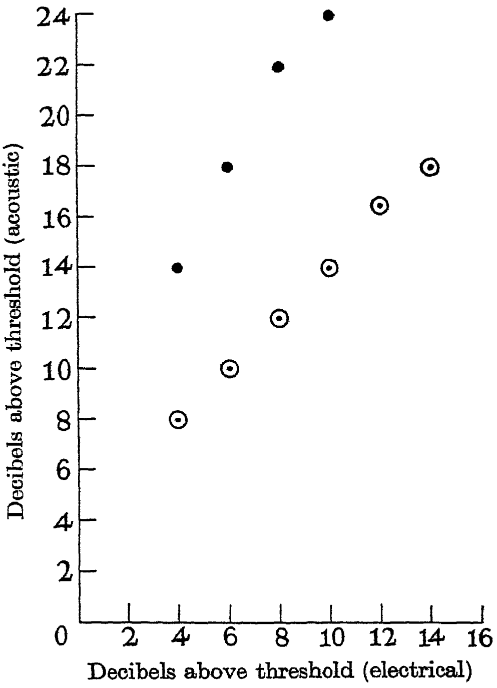


FIG. 4—Intensity difference (log I/I_0) sensitivity for acoustic and electrical stimulation. Observer C. S. H. 512 ●. 1024 ⊙.

hold at which any given subjective response is evoked by acoustic or electrical stimulation.

EXPERIMENTAL RESULTS

These may be described under three headings:

1—A comparison of the sensation levels above threshold at which beats were heard in response to reversal in phase of acoustic and electrical waves series of frequencies 512 and 1024 ~. Oscillographic records of the wave forms of the stimuli employed are shown in fig. 17, Plate 6. The results are given in figs 5, 6, 7 and 8.

2—A comparison of the sensation levels above threshold at which beats were heard in response to interruptions of 4 cycles duration in acoustic and electrical waves series of frequencies 512 and 1024 ~. Oscillographic records of the wave forms of the stimuli employed are shown in fig. 18, Plate 6. The interruptions were produced by removal of four of the spokes on the periphery of the disk of the photo-electric tone generator. Further adjustment of the resulting wave form was effected by suitable modification of the disk contour within the gap.

The results are given in figs. 9, 10, 11 and 12.

3—A comparison of the sensation levels above threshold at which beats were heard in response to interruptions of 10 ~ duration in acoustic and electrical wave series of frequencies 512 and 1024 ~. The interruptions were produced by the method described in the case of the 4 ~ interruptions. The results are given in figs. 13, 14, 15 and 16.

Summary of Experimental Results

A quantitative study has been made of the beats which are produced as the subjective responses to reversal in phase or transient interruption of a wave series, either acoustic or electrical, employed for excitation of the human cochlea.

For both acoustic and electrical modes of stimulation a close correspondence is shown to exist in the sensation levels at which such beats are heard.

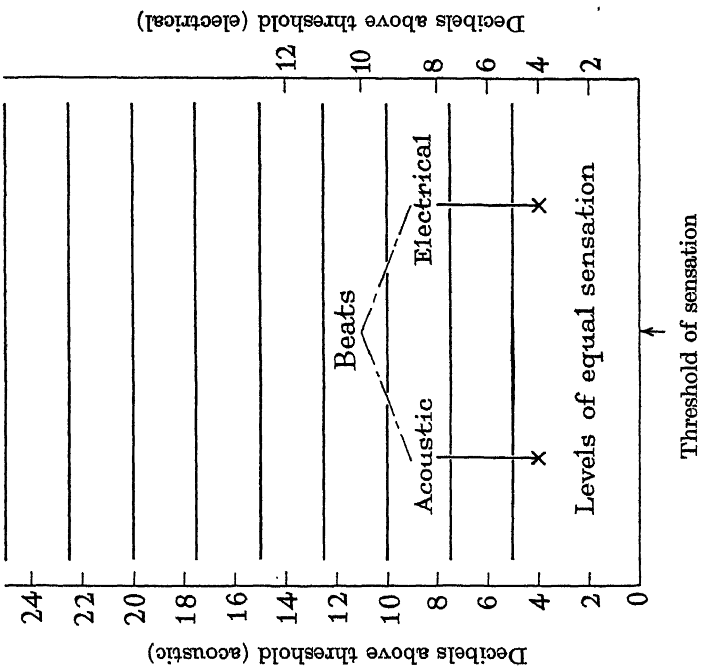


FIG. 6—Observer H. H. 1024 ~. Phase reversal beats appear at the levels indicated.

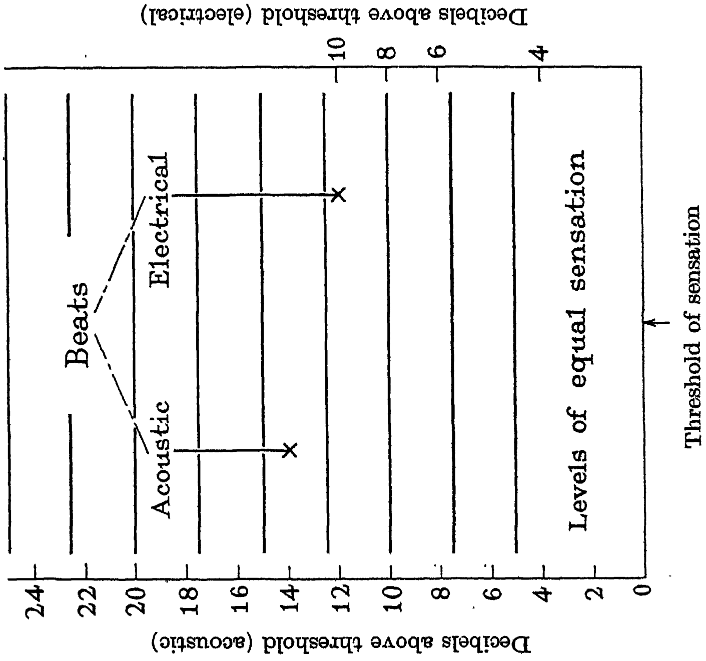


FIG. 5—Observer H. H. 512 ~. Phase reversal beats appear at the levels indicated.

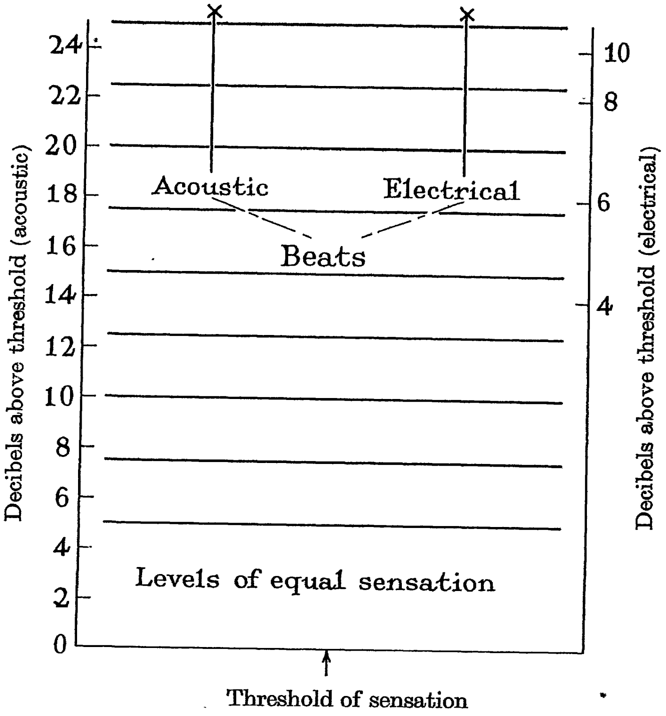


FIG. 7—Observer C. S. H. 512 ~. Phase reversal beats appear at the levels indicated.

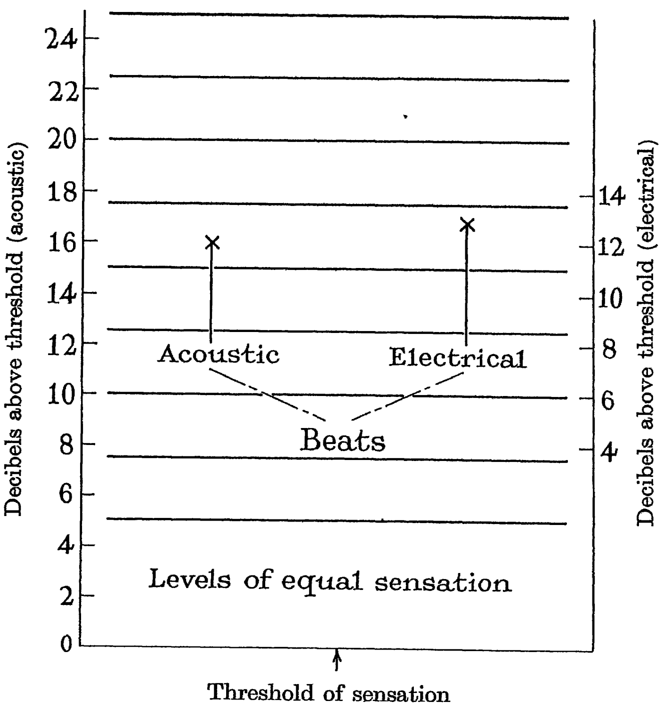


FIG. 8—Observer C. S. H. 1024 ~. Phase reversal beats appear at the levels indicated.

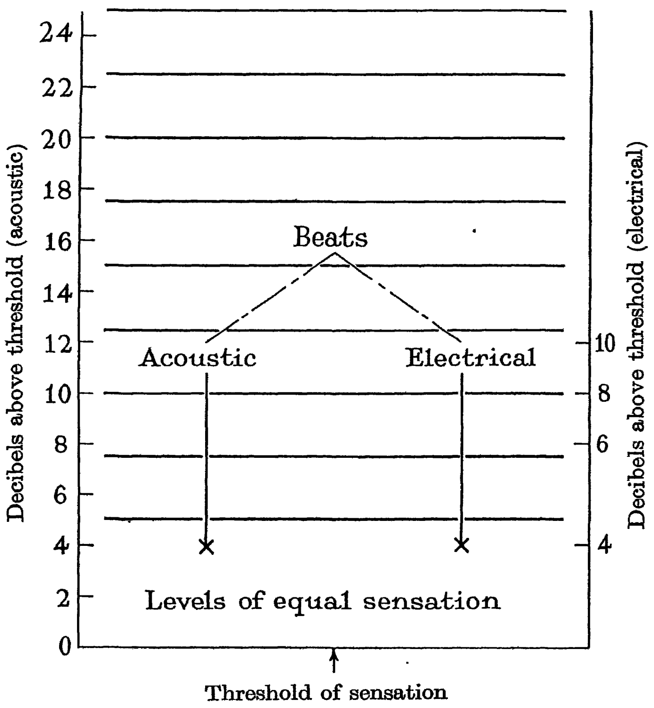


FIG. 9—Observer H. H. 512 ~. 4 ~ interruptions. Beats appear at the levels indicated.

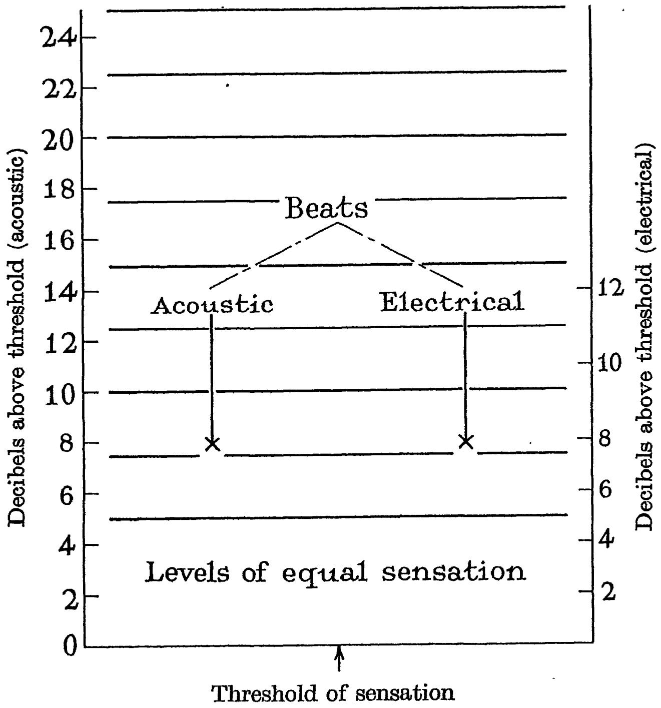


FIG. 10—Observer H. H. 1024 ~. 4 ~ interruptions. Beats appear at the levels indicated.

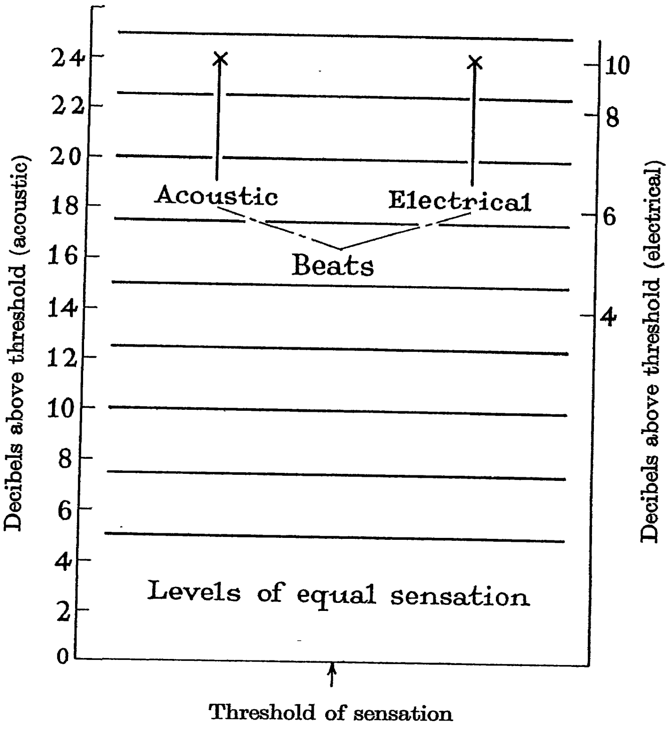


Fig. 11—Observer C. S. H. 512 ~. 4 ~ interruptions. Beats appear at the levels indicated.

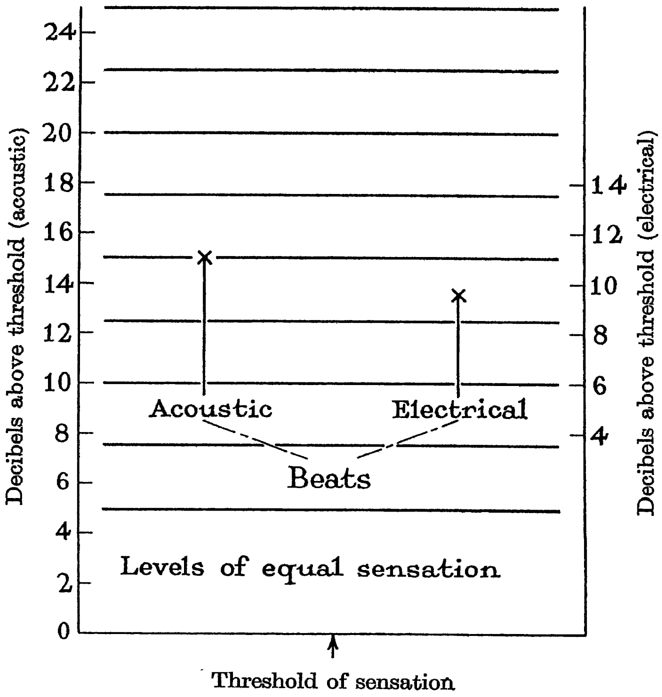


Fig. 12—Observer C. S. H. 1024 ~. 4 ~ interruptions. Beats appear at the levels indicated.

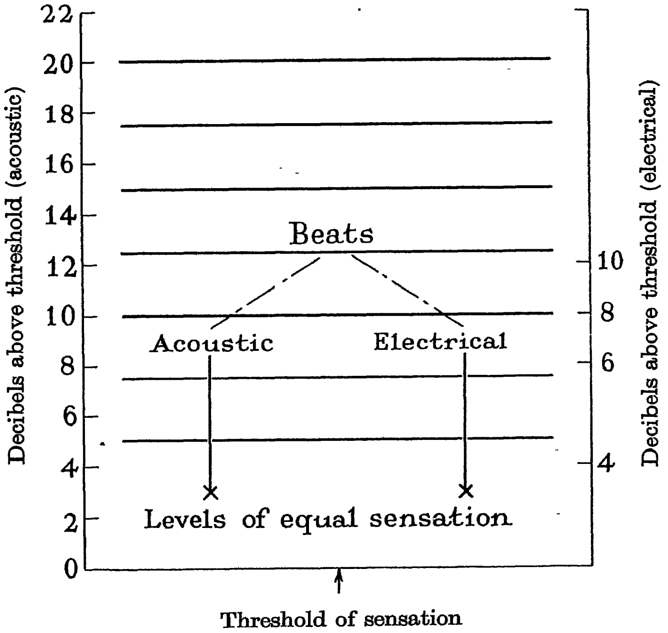


FIG. 13—Observer H. H. 512 ~. 10 ~ interruptions. Beats appear at the levels indicated.

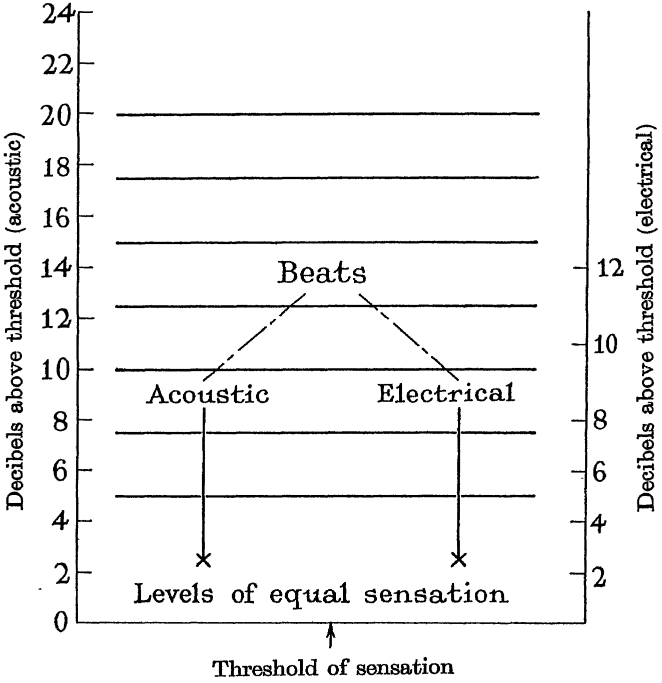


FIG. 14—Observer H. H. 1024 ~. 10 ~ interruptions. Beats appear at the levels indicated.

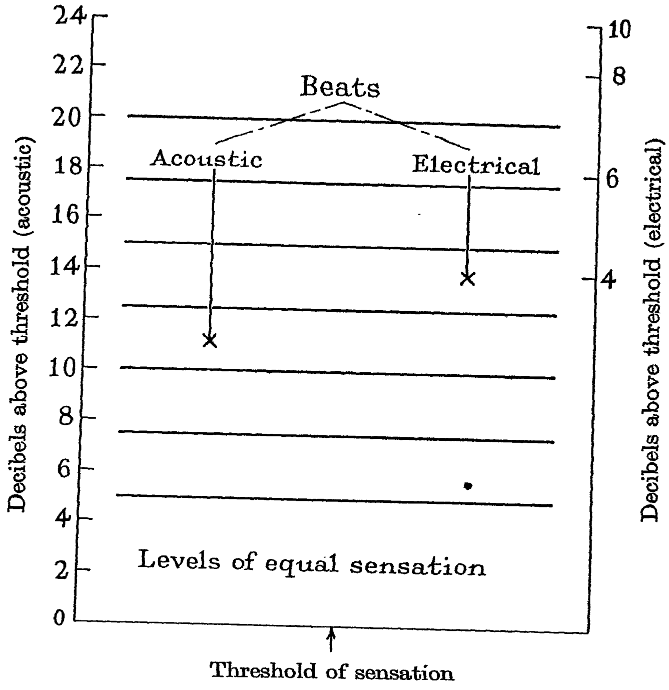


FIG. 15—Observer C. S. H. 512 ~. 10 ~ interruptions. Beats appear at the levels indicated.

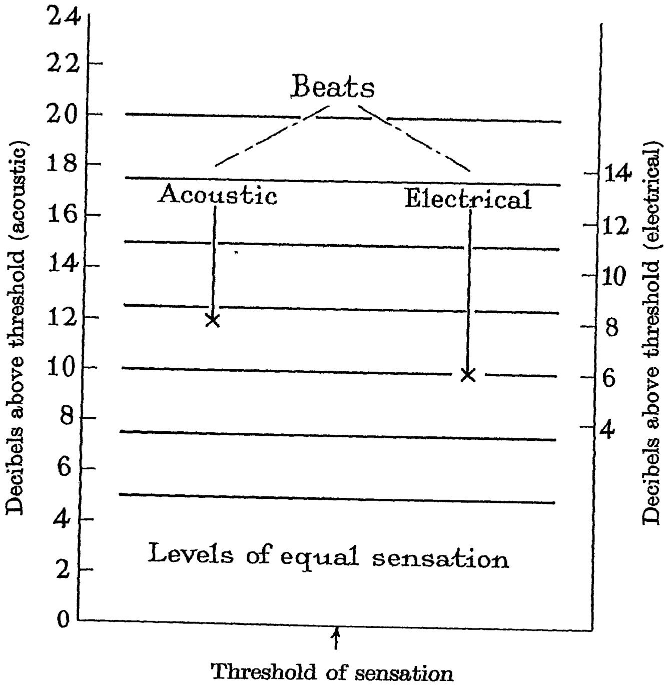


FIG. 16—Observer C. S. H. 1024 ~. 10 ~ interruptions. Beats appear at the levels indicated.

DISCUSSION

The line of argument upon which the experimental results here described have been interpreted may be restated as follows:

A reversal in phase or a transient interruption in a continuous musical tone brings about the sensation of a beat which has as its basis a transient arrest in the motion of certain resonant elements of the internal ear. The sensation level at which such a beat becomes apparent must depend in part upon the physical properties of the resonant elements certainly concerned in the case of acoustic stimulation and possibly in the case of electrical stimulation. A close correspondence of this level determined in the case of acoustic stimulation with that similarly determined in the case of electrical stimulation by a wave series of identical form has been demonstrated, and is considered to support the view that both types of excitation employ the common peripheral pathway represented by the moving parts of the internal ear. In so far, therefore, the findings must be considered to be in accord with the movement hypothesis of excitation by audiofrequency electrical stimulation. It must, however, be emphasized that although the evidence at present available would appear to support the view that the "phase reversal beat" has as its physiological basis a transient silent period in the electrical response of the auditory tracts, nevertheless the further possibility cannot as yet be excluded that other receptors are positively concerned by the phase reversal. It seems, for example, by no means impossible that the saccular end organs might respond in a positive sense to the transient pressure disturbance associated with a reversal in phase or a transient interruption of a continuous musical tone, and that this response might in fact play a part in the production of the resulting beat. So far, however, as this concerns the purpose of the present paper, it may be indicated that such a response on the part of the saccular or other extracochlear end organs is one in which movement of the elements of the internal ear would certainly be involved. Hence it would appear unlikely to affect the general interpretation which has been adopted of the present experimental results.

As regards the mechanism of transformation of electrical into mechanical energy, it seems likely that this may be effected upon the basis of some standing electrical field within the cochlea. The presence of such a field has already been suggested in explanation of the origin of those potential changes set up within the cochlea in response to sound waves which constitute the cochlear component of the Wever and Bray phenomenon. The locus of this field has been variously suggested by Davis (Davis and others

1934) as being the hair cells of Corti's organ, and by Hallpike and Rawdon-Smith (1934) as being the membrane of Reissner. It may be added that this relationship between the Wever and Bray phenomenon and the response of the ear to electrical stimulation has been previously suggested by Fromm, Nylen and Zottermann (1933) and would appear to conform well with the experimental facts.

SUMMARY

As previously described, reversal in phase of a continuous musical tone causes a beat to be heard by the human subject.

This beat has been shown to be due, at any rate in part, to a temporary arrest in the motion of the resonant elements of the internal ear.

In the present work, experimental treatment of this beat has been undertaken upon the lines of a flicker phenomenon, and a determination made for frequencies of 512 and 1024 ~ of the sensation levels above threshold at which the beat is heard.

Close correspondence is described between these levels and the corresponding levels similarly determined in the case of electrical stimulation of the ear by a wave series of identical form.

A similar correspondence is described in the case of beats caused by transient interruptions in tones of 512 and 1024 ~.

These findings are considered to support the movement hypothesis of excitation of the human ear by audio-frequency electrical stimulation.

A probable relationship is indicated between the transformation mechanism so involved and that underlying the origin of the cochlear component of the Wever and Bray phenomenon.

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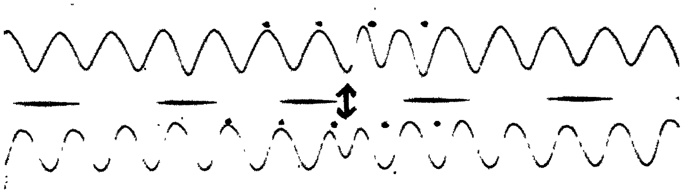

FIG. 17*a*

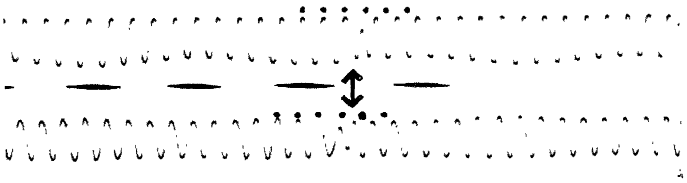

FIG. 17*b*

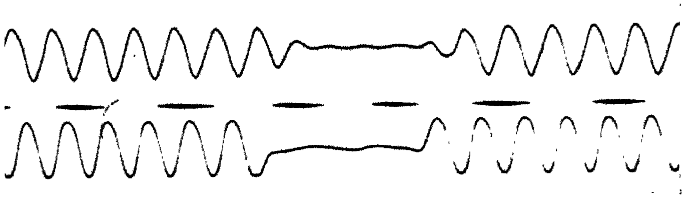

FIG. 18*a*

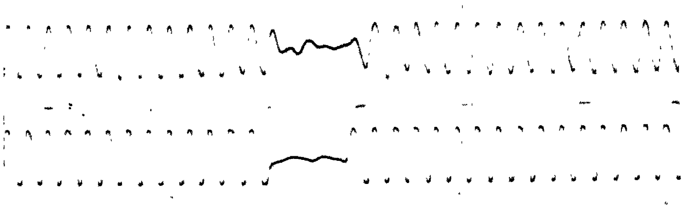

FIG. 18*b*

DESCRIPTION OF PLATE 6

FIG. 17—Oscillograph records (*a*, 512 ~; *b*, 1024 ~) showing reversal in phase of the wave series employed for acoustic and electrical stimulation. In each record, the upper tracing is the piezo-microphone record of the acoustic stimulus. The electrical stimulus is shown in the lower tracing. Arrows indicate the phase reversals. Time marker $\frac{1}{200}$ sec.

FIG. 18—Oscillograph records (*a*; 512 ~; *b*, 1024 ~) showing transient interruptions in the wave series employed for acoustic and electrical stimulation. In each record, the upper tracing is the piezo-microphone record of the acoustic stimulus. The electrical stimulus is shown in the lower tracing. Time marker $\frac{1}{200}$ sec.

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Enzymes of some Wood-Rotting *Polypores*

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1—INTRODUCTION

Very few *Polypores* have been studied with regard to their enzymes, though enzymes are the chief weapons with which these fungi invade the plant cells and bring about a very great amount of damage to logs, timbers, and trees. In 1895, Bourquelot and Herissey (1897) investigated the enzymes from the juice of *Polyporus sulphureus*, and in 1899, Czapek (1899) discovered the lignin-destroying enzyme in *Merulius lacrymans*. Two years later, Kohnstamm (1901) made some attempts to determine the enzymes of *Merulius lacrymans* and *Armillaria mellea*, but his results were inconclusive as the technique of enzyme chemistry then was not fully developed. Subsequently, Buller (1906) brought out a list of enzymes present in the expressed sap of the young fruit-bodies of *Polyporus squamosus* growing in nature. In recent years Zeller (1916), Schmitz (1920, 1921, 1925), Lutz

(1930, 1931), Schmitz and Zeller (1919), Nutman (1929), Yamano (1931), Venkatarayan (1936) and others have carried out important investigations in this line. From the literature cited above it appears that only a very few *Polypores* (about a dozen) have been studied with regard to their enzymic activity.

In the present paper the more important enzymes of eight species of wood-destroying *Polypores*, viz. *Polyporus ostreiformis*, *Polystictus hirsutus*, *Daedalea flavida*, *Polystictus sanguineus*, *Trametes lactinea*, *Polystictus leoninus*, *Trametes cingulata* and *Polyporus zonalis* have been studied in detail in artificial culture. The enzymic activity, both intracellular and extracellular, was measured at three different stages of growth, viz. (1) young vegetative mycelium, (2) mycelium about to bear fruits, and (3) mycelium with fruit-bodies. Side by side, the enzymes contained in mature fruit-bodies collected from nature were also studied. The nature of decay of mango-wood blocks inoculated with pure cultures of these *Polypores* was also studied. Incidentally, the change of pH values after growth, the production of fruit-bodies in artificial cultures and cultural characters in liquid medium were noted.

2—MATERIAL AND METHODS

Pure cultures of *Polypores* were obtained by the usual spore-culture method, and they were subcultured in 2 % malt-agar slants ($p\text{H} = 6{\cdot}8$) in tubes. The fungus-meal and the solution containing secreted enzymes were prepared in the following way:

To ensure comparable results all the *Polypores* were grown in 2 % malt-extract (liquid) medium with $p\text{H} = 6{\cdot}8$. A series of Erlenmeyer flasks containing 100 c.c. of this liquid medium were autoclaved at 115–120° C. for 20 min., and on cooling were inoculated with a mycelial transfer from the spore culture of the *Polypore* required. The fungus mats were taken out at three different stages of growth as stated previously, and were washed in running tap water followed by distilled water till they were free from the adhering medium. These mats were dried partially by being put between sheets of blotting paper and finally dried in a vacuum desiccator over sulphuric acid. They were weighed, finely powdered in a mortar and kept in a refrigerator for use as sources of intracellular enzymes. After taking out the mats, the liquid medium left behind was collected and measured. An aliquot part of this medium was filtered and preserved with a few drops of toluene in the refrigerator. This liquid medium contained the secreted enzymes, which will be called extracellular enzymes in all subsequent descriptions.

From the dry weight of the fungus mats and the volume of liquid medium we have calculated the quantity of the latter which corresponds to 1 g. of dry fungus mat. The intracellular and extracellular enzymes have been compared on this basis.

3—CULTURAL CHARACTERS

(*a*) *The nature of growth of different* Polypores *in liquid medium (malt extract)*

In liquid medium (2 % malt extract) in Erlenmeyer flasks and at room temperature (26–32° C.), *Polystictus hirsutus*, *P. sanguineus* and *Trametes cingulata* grow rather slowly and do not form a thick mat on the surface; they form a loose growth on the surface with a large amount of submerged mycelium. *Polyporus zonalis*, *P. ostreiformis*, *Polystictus leoninus*, *Trametes lactinea* and *Daedalea flavida*, on the other hand, grow very quickly and the liquid surface is generally covered with a thick mycelial mat before the small fruit-bodies appear here and there; there is also some amount of submerged mycelium. Clamp connexions were noticed in all of them (in both the aerial and submerged hyphae) with abundant crystals of calcium oxalate. A number of conidia were found in old cultures of *Polystictus hirsutus*, *P. sanguineus* and *Trametes cingulata*.

(*b*) *Production of fruit-bodies in culture*

In liquid medium (2 % malt extract) in flasks *Polyporus ostreiformis*, *Polystictus leoninus*, *P. hirsutus*, *P. sanguineus* and *Trametes cingulata* fruited quickly (15–20 days), while *Polyporus zonalis*, *Daedalea flavida* and *Trametes lactinea* produced small resupinate porous areas in 50–60 days.

In sterilized mango-wood block cultures in Roux tubes the fruit formation was much delayed; for instance, *Polyporus ostreiformis* fruited after 2 months and 18 days, *Polystictus leoninus* after 1 month and 4 days, *P. hirsutus* after 1 month and 20 days, *P. sanguineus* after 1 month and 12 days, *Trametes cingulata* after 5 months and 25 days, *Polyporus zonalis* after nearly 4 months, *Daedalea flavida* after 2 months and 4 days and *Trametes lactinea* after nearly 11 months.

(*c*) *Change of* pH *values after growth in liquid medium*

The initial pH value of the inoculated liquid medium (2 % malt extract) in each case was 6·8. After growth in the course of 50–60 days the pH value came down to 3·2 in the flasks with *Polyporus ostreiformis* and *Polystictus hirsutus*; the *Polystictus sanguineus* flask showed pH 4·2, *Daedalea flavida*

pH 4·4, *Trametes lactinea* pH 4·2, *Polyporus zonalis* pH 5, *Polystictus leoninus* pH 5·6 and *Trametes cingulata* showed pH 5·1.

4—NATURE OF DECAY IN INOCULATED WOOD-BLOCKS

The nature of microscopical and macroscopical changes in the inoculated mango-wood blocks has been described in detail in connexion with the enzyme ligninase (p. 205) in Table VIII under the headings of microscopical and macroscopical observations. It has been found that *Polystictus sanguineus*, *P. hirsutus* and *Daedalea flavida* produce *white rots* in the inoculated wood-blocks, thus belonging to the lignin-destroying group, while the rest produce more or less *brownish rots*, being principally cellulose-destroying fungi. In recent years there has been a good deal of controversy regarding the classification of wood decays into brown and white rots, as pointed out by Campbell (1930). We have, therefore, followed the results of microscopical findings by subsequent estimation of lignin in original and decayed wood, and we find that these results mutually agree. Campbell (1932) has further classified white rots on chemical grounds into three distinct groups, viz. (1) white rots in which lignin is attacked in the early stage and the attack on cellulose is delayed; (2) white rots in which cellulose is attacked in the early stage and the attack on lignin is delayed; (3) white rots in the early stage of which both lignin and cellulose are attacked but in varying proportions. From Table VIII it is clear that in at least two cases of white rots (by *Polystictus sanguineus* and *Daedalea flavida*) there was no delignification in the course of 2½–4 months, but a distinct sign of delignification was noticed after 6½ months. So, probably, they belong to the second group of white rots. In the progressive decay of ashwood by *Polyporus hispidus*, Cartwright and others (1936) have found that the lignin was not attacked at the immediate onset of decay, and that there was distinct evidence of attack after about 20 weeks (i.e. 5 months). In the white rot caused by *Polystictus hirsutus* delignification was evident from the beginning and it progressed much in the course of 5 months. Of course, at very advanced stages of decay it becomes difficult to differentiate one type of white rot from another by chemical means, the ultimate primary reactions being probably the same in all three groups of white rots, as remarked by Campbell (1932, p. 1836). We entirely agree with Campbell (1932) and Cartwright and others (1936) that "the white rot fungi are not consistent in the manner of their attack on wood and there is no general uniformity with regard to either the order or proportion in which the major wood components are decomposed".

5—ENZYMES PRESENT

The results of enzymic investigation together with the methods are given below:

In all cases estimations were carried out in duplicate and mean values taken. Side by side, control flasks both with the active enzymes without substrate and the inactivated enzymes (inactivated by autoclaving at 15 lb. pressure for 15 min.) were kept to correct for any error due to hydrolysis or the presence of any reducing substances, i.e. the figures show the real values obtained by the action of the enzyme on the substrate. The amount of correction was practically negligible.

Invertase—The reaction mixture was composed as follows:

(*a*) For intracellular enzymic study: 20 c.c. of 0·2 % saccharose, 10 c.c. citrate buffer of *p*H 4·4, 7 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 20 drops of 0·2 % saccharose, 10 c.c. citrate buffer of *p*H 4·4, 5 c.c. distilled water, 2 c.c. of extracellular enzyme and 6 drops of toluene.

To facilitate comparison the total digested volumes in both (*a*) and (*b*) were kept the same. The flasks containing the reaction mixtures were stoppered and incubated at 37° C. for 7 days, after which an aliquot part was taken out, and the reducing sugar was estimated by Shaffer and Hartmann's (1921) micro-method. The mean results of duplicate estimations are given in Table I.

It appears from Table I that the amount of the invertase enzyme, both intracellular and extracellular, is greatest in *Polyporus ostreiformis* and

TABLE I

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	16·03	15·43	12·73	3·25
Polystictus hirsutus	11·13	10·14	10·03	2·51
Polystictus sanguineus	11·67	10·65	10·41	2·39
Daedalea flavida	2·74	2·42	2·08	0·82
Trametes lactinea	4·44	3·81	3·44	1·02
Polystictus leoninus	12·36	11·25	11·05	1·75
Trametes cingulata	6·62	5·89	4·58	1·12
Polyporus zonalis	10·50	9·85	8·56	2·23

TABLE I—(*continued*)

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing enzymic solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	198·40	181·10	164·00
Polystictus hirsutus	154·60	129·81	106·41
Polystictus sanguineus	96·27	94·62	69·53
Daedalea flavida	23·79	22·51	21·90
Trametes lactinea	23·92	22·68	22·59
Polystictus leoninus	160·40	140·54	130·58
Trametes cingulata	24·75	23·52	23·01
Polyporus zonalis	150·32	124·56	104·63

the least in *Daedalea flavida*. There is a progressive diminution of activity from the vegetative to the fruiting stage, and the activity of the natural sporophore is less than that of the sporophore in artificial culture. It is also seen that the extracellular enzymic activity is much greater.

Raffinase—The reaction mixture consisted of the following:

(*a*) For intracellular enzymic study: 20 c.c. 0·2 % raffinose, 10 c.c. citrate buffer of *p*H 4·4, 7 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 20 c.c. of 0·2 % raffinose, 10 c.c. citrate buffer of *p*H 4·4, 5 c.c. distilled water, 2 c.c. enzyme solution and 6 drops of toluene. The remaining procedure was the same as in the case of invertase and the results obtained are given in Table II.

TABLE II

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	7·85	7·57	6·83	1·25
Polystictus hirsutus	7·58	6·42	6·14	0·25
Polystictus sanguineus	7·24	6·89	6·42	0·41
Daedalea flavida	5·94	4·48	3·87	0·92
Trametes lactinea	5·35	5·28	4·27	1·12
Polystictus leoninus	7·62	7·45	6·87	1·54
Trametes cingulata	5·36	4·85	4·15	1·04
Polyporus zonalis	6·45	5·83	5·75	1·21

TABLE II—(*continued*)

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing enzyme solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	119·60	111·50	103·20
Polystictus hirsutus	74·71	68·44	66·25
Polystictus sanguineus	128·30	127·00	127·00
Daedalea flavida	46·19	45·05	44·84
Trametes lactinea	44·42	42·49	43·19
Polystictus leoninus	75·72	70·95	68·48
Trametes cingulata	47·26	46·34	45·68
Polyporus zonalis	72·65	67·78	66·08

It is seen from Table II that *Polyporus ostreiformis* is the most active and *Trametes lactinea* the least. As in the case of invertase, the extracellular enzyme solution is much more active and there is a fall of activity from the vegetative to the fruiting stage. The natural sporophore is also less active than the artificially grown one.

Maltase or α-glucosidase—The reaction mixture consisted of the following:

(*a*) For intracellular enzymic study: 20 c.c. of 0·1 % maltose, 10 c.c. phosphate buffer of *p*H 6·2, 7 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 20 c.c. of 0·1 % maltose, 10 c.c. of phosphate buffer of *p*H 6·2, 5 c.c. distilled water, 2 c.c. enzyme solution and 6 drops of toluene.

The remaining procedure was the same as in the previous case and the increase in reducing sugar formed was estimated as before by Shaffer and Hartmann's method. See Table III.

In this case also the activity of *Polyporus ostreiformis* is greatest. There is also a gradual fall of activity as before, and the extracellular enzyme solution is much more active.

Amylase—The reaction mixture was made up as follows:

(*a*) For intracellular enzymic study: 20 c.c. of 1 % starch solution (Merck's soluble starch), 10 c.c. phosphate buffer of *p*H 4·9, 7 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 20 c.c. of 1 % starch solution, 10 c.c. phosphate buffer of *p*H 4·9, 5 c.c. distilled water, 2 c.c. enzyme solution and 6 drops of toluene.

TABLE III

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	7·58	6·83	6·28	1·04
Polystictus hirsutus	3·01	1·60	1·45	0·34
Polystictus sanguineus	2·62	2·24	1·98	0·81
Daedalea flavida	6·21	5·49	5·28	1·12
Trametes lactinea	5·29	3·85	3·62	0·94
Polystictus leoninus	3·25	2·84	2·25	0·64
Trametes cingulata	6·30	4·57	4·08	1·21
Polyporus zonalis	2·72	1·55	1·32	0·45

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing enzyme solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	46·93	37·72	34·60
Polystictus hirsutus	11·87	10·77	9·21
Polystictus sanguineus	12·34	11·00	9·67
Daedalea flavida	19·78	19·04	5·22
Trametes lactinea	18·63	17·23	16·43
Polystictus leoninus	12·72	11·50	10·76
Trametes cingulata	19·52	18·67	17·85
Polyporus zonalis	10·98	9·65	8·75

The reaction flasks with the control ones were incubated at 37° C. for 24 hr., after which the reducing sugar formed was estimated as in previous cases. See Table IV.

Here the extracellular enzyme of *Polyporus ostreiformis* possessed the maximum activity. As in previous cases the extracellular enzyme solution was much more active and a gradual fall in activity from the vegetative to the fruiting stage was noticed.

Emulsin or β-glucosidase—The reaction mixture consisted of the following:

(*a*) For intracellular enzymic study: 10 c.c. of 1 % amygdalin, 2 c.c. 0·1 N acetate buffer, 5 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 10 c.c. of 1 % amygdalin, 2 c.c.

0·1 N acetate buffer, 3 c.c. distilled water, 2 c.c. enzyme solution and 6 drops of toluene.

Table IV

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	34·13	32·76	24·57	6·12
Polystictus hirsutus	7·64	6·01	4·91	1·24
Polystictus sanguineus	35·53	34·40	33·37	2·53
Daedalea flavida	28·73	27·16	25·21	6·15
Trametes lactinea	18·84	18·09	17·65	4·41
Polystictus leoninus	21·92	17·58	15·78	3·92
Trametes cingulata	18·79	17·56	17·15	4·25
Polyporus zonalis	6·85	5·89	4·69	1·12

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 37 c.c. containing enzyme solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	470·32	462·19	345·10
Polystictus hirsutus	213·49	191·24	169·74
Polystictus sanguineus	260·03	257·37	245·71
Daedalea flavida	103·81	102·44	101·77
Trametes lactinea	100·26	98·58	98·35
Polystictus leoninus	313·51	295·87	275·69
Trametes cingulata	101·72	99·43	98·59
Polyporus zonalis	208·57	180·18	160·83

The reaction flasks with the control ones were incubated at 37° C. for 7 days, after which an aliquot part was taken out and the HCN was driven out by evaporation, and finally the reducing sugar was estimated by Shaffer and Hartmann's method.

It is evident from Table V that all these *Polypores* contain sufficient glucoside-splitting enzyme. Among them *Polystictus sanguineus* appeared to be the most active. In this case also the extracellular enzyme solution was much more active and there was a diminution of activity from the vegetative to the fruiting stage.

Hemicellulase—As substrate, finely powdered endosperm of date seeds (*Phoenix dactylifera*), used by the majority of investigators, was used in the present investigation.

TABLE V

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 17 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	24·84	21·84	20·88	5·22
Polystictus hirsutus	23·34	21·77	16·65	4·12
Polystictus sanguineus	24·60	22·93	21·05	1·66
Daedalea flavida	13·40	11·94	10·85	2·41
Trametes lactinea	8·21	7·23	6·89	1·53
Polystictus leoninus	24·40	20·40	17·86	4·45
Trametes cingulata	8·45	7·45	6·45	1·60
Polyporus zonalis	17·54	16·87	15·76	3·85

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 17 c.c. containing enzyme solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	143·66	128·79	123·19
Polystictus hirsutus	118·28	109·51	105·38
Polystictus sanguineus	150·83	147·42	146·06
Daedalea flavida	40·20	38·02	36·90
Trametes lactinea	61·41	58·96	58·70
Polystictus leoninus	120·50	111·56	107·48
Trametes cingulata	58·36	57·78	55·87
Polyporus zonalis	107·76	104·68	101·56

The reaction mixture was composed as follows:

(*a*) For intracellular enzymic study: 0·5 g. powdered date-seed meal, 15 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 0·5 g. powdered date-seed meal, 10 c.c. distilled water, 5 c.c. enzyme solution and 6 drops of toluene.

The reaction flasks with the control ones were incubated at 37° C. for 21 days and the reducing sugar was estimated at the end of that period.

Date seeds (*Phoenix dactylifera*) were thoroughly washed and were scraped with emery paper to remove their outer coats. They were then rinsed in distilled water, cracked open and the embryos cut out. The hemicellulose (endosperm) thus obtained was autoclaved in distilled water at 15 lb. pressure for 20 min. to deactivate all enzymes as well as to kill any micro-organisms present. Van Tieghem cells were prepared and very thin slices of hemicellulose were suspended in hanging drops of both intracellular and extracellular enzyme solutions along with the control (sterilized distilled water). To each cell a few drops of toluene was added as an antiseptic, and at the bottom of each cell a small quantity of sterilized distilled water was placed to prevent evaporation. After 1 month, erosion of the hemicellulose was noticed in all drops of enzymes but not in the distilled water (control). After about a month and a half the erosion was very distinct, at places only some granular substances and a few fragments were left. Thus, it is evident from Table VI as well as from the experiments with Van Tieghem cells that hemicellulase is present in all the eight *Polypores*.

Table VI

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 15 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	1·64	1·37	1·16	0·24
Polystictus hirsutus	1·23	1·02	0·85	0·26
Polystictus sanguineus	1·50	1·39	1·30	0·41
Daedalea flavida	1·30	1·09	1·00	0·24
Trametes lactinea	1·64	1·50	1·38	0·32
Polystictus leoninus	1·42	1·21	0·88	0·21
Trametes cingulata	1·37	1·15	0·84	0·24
Polyporus zonalis	1·15	0·98	0·81	0·25

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 15 c.c. containing enzyme solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	9·49	8·26	6·62
Polystictus hirsutus	4·10	3·41	2·93
Polystictus sanguineus	4·40	3·36	3·34
Daedalea flavida	3·44	2·66	2·57
Trametes lactinea	2·93	2·54	2·48
Polystictus leoninus	4·75	3·65	3·15
Trametes cingulata	3·48	2·76	2·51
Polyporus zonalis	3·78	3·12	2·78

Cellulase—The reaction mixture was composed as follows:

(*a*) For intracellular enzymic study: 10 c.c. of finely pulped filter-paper-cellulose suspension in water, 5 c.c. distilled water, 0·1 g. fungus-meal and 6 drops of toluene.

(*b*) For extracellular enzymic study: 10 c.c. of the above cellulose suspension, 5 c.c. of enzyme solution and 6 drops of toluene.

The reaction flasks were incubated at 37° C. for 21 days and then the reducing sugar estimated as in previous cases.

TABLE VII

A—*Intracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 15 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	3·41	2·93	2·77	0·62
Polystictus hirsutus	2·32	1·91	1·64	0·41
Polystictus sanguineus	3·00	2·53	1·98	0·57
Daedalea flavida	2·07	1·40	1·12	0·34
Trametes lactinea	2·07	1·46	1·27	0·32
Polystictus leoninus	2·45	2·21	1·95	0·45
Trametes cingulata	2·12	2·01	1·59	0·51
Polyporus zonalis	1·85	1·65	1·51	0·49

B—*Extracellular enzymic activity*

Figures given in mg. of reducing sugar formed in 10 c.c. of the total digested volume of 15 c.c. containing enzyme solution corresponding to 0·1 g. of fungus-meal.

Species	Young vegetative stage	About-to-fruit stage	Fruiting stage
Polyporus ostreiformis	16·38	13·65	12·76
Polystictus hirsutus	10·24	6·83	6·14
Polystictus sanguineus	5·68	4·12	3·52
Daedalea flavida	4·12	2·76	2·63
Trametes lactinea	3·44	2·80	2·76
Polystictus leoninus	11·72	8·79	7·65
Trametes cingulata	4·43	3·42	2·85
Polyporus zonalis	8·76	5·79	5·03

Cellulase is, thus, seen to be present in all the *Polypores* studied, but the activity of cellulase as well as of hemicellulase is not so marked as that of other carbohydrates. This point will be dealt with in the discussion.

Lactase and Zymase—All the *Polypores* were tested for these two enzymes which were found to be absent.

Pectinase—To study the activity of this enzyme, uniformly thin sections of potato tubers were cut off and kept immersed in the extracellular enzyme solution and in aqueous suspension of enzymes from the fungus-meal in closed vessels, and they were examined from time to time. All the types showed the presence of pectinase. The time required to cause the loss of coherence of cells was between 20 and 25 hr. and no perceptible difference in activity in different types of *Polypores* could be noticed. For accurate quantitative work the enzyme requires to be purified and concentrated, which, however, could not be carried out in the present case.

Ligninase—The hadromal reaction of Czapek to detect and estimate the activity of lignin-destroying enzymes being not very suitable, the action of *Polypores* on wood-lignin was studied in the following way:

Dead mango-wood blocks were soaked with water by being alternately put into cold and hot water for 3 days; they were then placed within Roux tubes and were sterilized in a Koch's sterilizer at 100° C. for 20 min. for three successive days, and afterwards were inoculated with these eight *Polypores*. Control Roux tubes containing representative sterilized sample blocks were kept side by side. The microscopical structure and the lignin content of the control wood blocks were determined at the beginning. Periodically, the lignin content of all the wood blocks was estimated and the microscopical examination of the inoculated wood blocks was compared with that of the control wood blocks. The lignin was estimated by a mixture of 18 % hydrochloric and 72 % sulphuric acids as described by Norman (Schwalbe's method) (1929). The results are given in Table VIII.

It appears from the figures obtained by the chemical analyses that the percentage of lignin (on dry basis in residual wood) in wood blocks attacked with *Polystictus sanguineus*, *Daedalea flavida* and *Polystictus hirsutus* is less than that of the control wood. This indicates that the lignin was attacked and the rate of decomposition of lignin was greater than the other cell constituents, thereby decreasing its percentage; hence it can reasonably be concluded that these *Polypores* are to a great extent lignin-destroying. In the case of wood blocks attacked with four of the remaining *Polypores* (namely, *Polyporus ostreiformis*, *P. zonalis*, *Polystictus leoninus* and *Trametes cingulata*), the percentage of lignin on dry basis in residual wood increased, which indicates that even if there was any destruction in lignin in these cases, the rate was very slow in comparison with the rate of destruction of other constituents of the wood blocks. They, therefore, are not active lignin destroyers and possibly belong to the cellulose-destroying group. It will be seen that the microscopical examination of the wood blocks also agrees in general with the findings of the chemical analyses.

TABLE VIII—ACTION OF *POLYPORES* ON MANGO-WOOD BLOCKS

Set	Species	Period of incubation	Macroscopical observation	Microscopical observation	Amount of lignin (by chemical analysis) %
1st	Control (sterilized mango-wood blocks in Roux tubes)				30·9 on dry basis
,,	*Polystictus hirsutus*	3 months	The colour of the wood changed from deep brown to whitish, the wood thus being white rotted. The wood has softened sufficiently	In some wood cells the lignin has completely disappeared, in some partially, and in others there is no loss of lignin. The cells towards the periphery show greater loss of lignin. In comparison with the vessels and medullary ray cells the wood cells show much greater delignification. In one or two vessels delignification is noticed, while some medullary ray cells exhibit greater delignification. This process of delignification, as it appears at present, is gradual. The cells of the pith region of the infected as well as healthy wood show a number of brown gummy drops. The old hyphae are without clamps and not constricted. The bore holes are large. The nature of rot is something like mottling, as described by Hubert (1931, p. 375)	27·4 on dry basis in residual wood
		5 months	As above	As above, but delignification was more prominent	22·9 on dry basis in residual wood
,,	*Polyporus ostreiformis*	3 months	The colour remained like that of the control but the wood has become softer and lighter	The order of attack is like that of *Polystictus hirsutus*, i.e. first wood cells, then medullary ray cells, and finally vessels are attacked. The nature of rot in longitudinal strips is quite different from that of *Polystictus hirsutus*. Almost all the wood and medullary ray cells show the presence of gummy drops. Apparently no delignification. Old hyphae are not constricted and are without any clamps, the bore holes are large	36·9 on dry basis in residual wood
		5 months	As above	Almost as above	39·9 on dry basis in residual wood
2nd	Control (sterilized mango-wood blocks in Roux tubes)				27·2 on dry basis
,,	*Polystictus sanguineus*	2½ months	Wood has become soft and brittle. The colour of the wood has become yellowish	Great effect; medullary ray cells, wood cells and vessels all affected, but there is no prominent sign of delignification. Hyphae with clamps and not constricted, no gum drops	24·6 on dry basis in residual wood
		6½ months	Wood has become brittle, very soft and light. The colour of the wood has become whitish, the wood thus being white rotted	Both medullary ray cells and wood cells completely, and vessels partially, delignified. Hyphae with clamps and not constricted. No gum drops	23·9 on dry basis in residual wood
,,	*Daedalea flavida*	4 months	Wood remains hard as before. The colour has become a little yellowish when compared with the control	Medullary ray cells affected, the wall has become thinner but there is no delignification as vessels and wood cells remain quite unaffected. Hyphae with clamps and not constricted, hence bore holes are probably large. There is exudation of gum drops here and there, but the control has gum drops also	28·4 on dry basis in residual wood

		6½ months	Wood has become very light, soft and brittle. The colour of the wood is changed to white (the wood thus being white rotted) while the colour of the control wood is dirty brown	Hyphae with distinct clamps and constricted, no gum drops. Medullary ray cells have become thinner and they have been broken at many places and are the most highly delignified. Wood cells are also affected though not so highly as the medullary ray cells. Wood cells are slightly delignified. The walls of the vessels have become thinner but retain the yellow colour in chloro-zinc iodine solution	22·6 on dry basis in residual wood
”	*Trametes lactinea*	2½ months	Wood remains as hard as before. There is no change of colour	No effect, no delignification. Hyphae with clamps and not constricted. Gum drops (round) here and there but they are also present in the control	28·8 on dry basis in residual wood
		6½ months	Wood has become very light, soft and brittle. The colour of the wood has changed to yellowish while the colour of the control is dirty brown	Medullary ray cells greatly eaten away, wood cells are also affected (have become thinner) though not so highly as medullary ray cells. Vessels almost unaffected but, at places where hyphae are found abundantly, vessels have been greatly affected. Exudation of gum drops copious. Hyphae with clamps and not constricted. A case of apparently *no delignification*	33·5 on dry basis in residual wood
3rd	Control (sterilized mango-wood blocks in Roux tubes)				26·8 on dry basis
”	*Polyporus zonalis*	4½ months	Wood has become very light and brittle, the colour of the wood has remained brownish—almost like that of the control	Wood cells, vessels and medullary ray cells have become thinner. Exudation of gum drops copious. Hyphae with clamps and not constricted	27·9 on dry basis in residual wood
		6 months, 24 days	As above	No delignification. Tracheids and medullary ray cells have become thinner. Exudation of gum drops copious. Hyphae with clamps and not constricted	28·9 on dry basis in residual wood
”	*Trametes cingulata*	4½ months	Wood has become lighter though not so brittle. The colour of the wood is brownish like that of the control	Wood cells and vessels slightly delignified, medullary ray cells have become thinner. No gum drops. Hyphae with clamps and not constricted	25·1 on dry basis in residual wood
		6 months, 24 days	Wood has become lighter though not brittle. The colour of the wood has changed to whitish	Slight delignification. Tracheids greatly affected, medullary ray cells have become thinner, though not delignified. No gum drops. Hyphae with clamps and not constricted	25·2 on dry basis in residual wood
”	*Polystictus leoninus*	4½ months	Wood has become very light, soft and brittle. The colour of the wood has changed to yellowish	No delignification. Medullary ray cells and wood cells have become thinner. Vessels affected here and there. Gum drops present. Hyphae with clamps and not constricted	37·1 on dry basis in residual wood
		6 months, 24 days	As above	As above	37·8 on dry basis in residual wood

Lipase—The lipolytic activity was estimated by the method of Kanitz (1925) and the results obtained are given in Table IX. No activity was found in extracellular enzyme solution, probably because the concentration was too small to be detected. The reaction flasks were incubated at 37° C. for 7 days.

TABLE IX

Figures given in c.c. of 0·04 N KOH for the whole digested mass containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	0·52	0·45	0·38	0·14
Polystictus hirsutus	0·35	0·28	0·28	0·12
Polystictus sanguineus	0·65	0·58	0·49	0·25
Daedalea flavida	0·58	0·52	0·47	0·16
Trametes lactinea	0·41	0·35	0·32	0·15
Polystictus leoninus	0·45	0·32	0·28	0·12
Trametes cingulata	0·43	0·37	0·34	0·14
Polyporus zonalis	0·31	0·27	0·25	0·10

Proteolytic enzymes—The proteolytic enzyme activity was studied by taking 2 % peptone or 2 % egg albumin as substrate. With peptone, slight activity was found but with albumin the activity was negligible. Here also no activity was found in the extracellular enzyme solution. The reaction mixture was made up as follows:

5 c.c. of 2 % peptone, 5 c.c. citrate buffer of *p*H 5, 2 c.c. distilled water, 0·1 g. fungus-meal and a few drops of toluene. The flasks were incubated at 37° C. for 24 hr., and afterwards 2 c.c. were titrated with 0·04 N alcoholic KOH according to the method of Willstätter and Waldschmidt-Leitz (1921); the results obtained are given in Table X.

TABLE X

Figures given in c.c. of 0·04 N KOH for 2 c.c. of the total digested volume containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	0·42	0·38	0·25	0·15
Polystictus hirsutus	1·66	0·80	0·55	0·20
Polystictus sanguineus	0·45	0·35	0·32	0·15
Daedalea flavida	0·52	0·48	0·36	0·12
Trametes lactinea	0·38	0·25	0·22	0·10
Polystictus leoninus	0·82	0·75	0·65	0·17
Trametes cingulata	0·42	0·28	0·24	0·11
Polyporus zonalis	0·75	0·64	0·58	0·14

Catalase—The catalase activity was studied in the following way. The extracellular enzyme solution, however, showed no activity. The reaction flasks contained the following:

20 c.c. of 1 % H_2O_2, 10 c.c. of phosphate buffer of *p*H 6·8, 7 c.c. of distilled water, 0·1 g. of fungus-meal; they were incubated at room temperature for 2 hr., after which they were titrated with 0·1 N $KMnO_4$ in presence of sulphuric acid. The figures obtained are given in Table XI.

TABLE XI—INTRACELLULAR ENZYMIC ACTIVITY

Figures given in c.c. of 0·1 N $KMnO_4$ for 10 c.c. of the total digested volume of 37 c.c. containing 0·1 g. of fungus-meal.

Species	Young vegetative mycelium	Mycelium about to fruit	Mycelium containing fruit-bodies	Fruit collected from nature
Polyporus ostreiformis	6·50	7·25	7·00	6·31
Polystictus hirsutus	6·50	6·00	7·00	6·30
Polystictus sanguineus	7·50	8·10	8·25	7·50
Daedalea flavida	4·75	5·25	5·35	6·10
Trametes lactinea	3·75	3·95	3·90	3·68
Polystictus leoninus	7·40	7·80	7·90	7·57
Trametes cingulata	4·30	4·35	4·60	3·98
Polyporus zonalis	4·70	4·90	5·60	6·04

It appears that the catalase activity slightly increases from the vegetative to the fruiting stage. This has also been noted by others.

Laccase—The activity of laccase was tested with Guaiac tincture. It was present in detectable quantity (both intracellular and extracellular) in *Polystictus sanguineus, Daedalea flavida* and *Trametes lactinea,* slightly in *Polystictus leoninus* and *Polyporus zonalis,* and absent in the others. No quantitative experiment was carried out with this enzyme.

6—DISCUSSION

Most of the previous enzyme studies of Polyporaceae deal with intracellular enzymes alone; the majority of the workers have made only qualitative estimation of enzymes and they determined the enzymic activity only at the young vegetative state in culture flasks. No doubt the enzymes are then very active, as they are secreted from the tips of the actively growing hyphae, but a comparison of the activity of the enzymes at different stages of growth up to the formation of fruit-bodies would be useful. In only one case (of *Lenzites sepiaria*) Zeller (1916) has shown that most of the enzymes found in the vegetative state, excepting oxidase, are scanty or absent in the sporophore. In the present work we have almost invariably compared the enzymic activity, both qualitatively and quantitatively, at three different stages of growth, viz. (*a*) young vegetative stage, (*b*) old mycelial (about-to-fruit) stage, and (*c*) fruiting stage; side by side, the intracellular enzymes of sporophores growing in nature during the rains on logs, stumps, etc., have been estimated. There is a regular progressive decline in activity in the majority of cases (except that of catalase) as the species in culture passes from the young stage ultimately to the fruiting stage, while in the fruits formed in nature the activity is considerably lower. In all cases excepting catalase and proteolytic enzymes the amount of extracellular enzymes was found to be larger than the corresponding intracellular ones. Of course, the quality and quantity of enzymes will vary with each species: for instance, the diastatic activity of *Polyporus ostreiformis* is about five times as great as that of *Polystictus hirsutus*, while the corresponding activity of maltase is about thrice as great. So, special stress has been laid on the determination of both intracellular and extracellular enzymes and their qualitative as well as quantitative estimation at three different stages of growth. Kohnstamm in 1901 started with the same idea of the determination of enzymes of some *Polypores* at the four above-mentioned stages, but he could not get any comparable results.

It is well known that the secretion of enzymes varies qualitatively and quantitatively according to the nature of the medium of the fungus. Having used malt-extract medium (2 % with *p*H 6·8) throughout, we had the predominance of carbohydrases; there being little cellulose, hemicellulose, pectin, lignin, etc. in this medium, the activity of cytohydrolysing enzymes was much less.

It would appear from our results that the *Polypores* contain and secrete both α and β glucoside-splitting enzymes. This is expected, as the *Polypores* decompose complex carbohydrates containing α and β linkages. The preponderance of β glucosidase (emulsin) over the α glucosidase (maltase) is possibly due to the fact that cellulose, which is decomposed by the *Polypores*, contains β linkages. While all the other enzymes like invertase, amylase, cellulase, maltase, etc. are secreted externally into the medium, the catalase is found only as an intracellular enzyme. This may be due to the fact that while the function of extracellular enzymes is to convert the food materials into available form, the catalase plays its role principally in the respiration of the fungi.

We should bear in mind that in a living organism enzymes never act alone in a pure state, they all act together; and, moreover, the external factors play a possibly predominating role as pointed out by Falck in (1935).

He thinks that the enzymes may be the heredity factor liable to be acted upon by the environment in any particular case.

In connexion with enzymes, especially in truly parasitic and hemiparasitic fungi, one should also consider the possibility that enzymes may sometimes be prevented from acting by forces exerted by the living host cell, a type of chemical and mechanical resistance offered by the plant, as pointed out by Brown (1934); in such cases there is no attack. Corner (1935) has shown that on an inappropriate host the early stages of penetration by a mildew parasite are the same as on the proper host, i.e. the cuticle is pierced mechanically. The penetration-process, however, usually develops no further in the inappropriate host and is probably killed by toxic substances in the "host" cell. Brown remarks (1934) that in some cases "the cell-wall substance of the plant is not hydrolysable by the enzymic apparatus of the fungus. As the best evidence available indicates that the protoplast-destroying agent only functions after previous attack on the cell-wall by the enzyme, there is likewise no attack on the living cells. Immunity, thus, ultimately rests upon the composition of the cell-wall". Herein lies the importance of knowledge of the chemistry of the cellulose, hemicellulose, pectin, lignin and similar substances.

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7—SUMMARY

1. The physiology of eight wood-rotting *Polypores* (*Polyporus ostreiformis*, *P. zonalis*, *Polystictus hirsutus*, *P. sanguineus*, *P. leoninus*, *Trametes cingulata*, *T. lactinea*, *Daedalea flavida*) has been studied regarding their enzymic action.

2. To ensure comparable results one particular medium of a definite concentration, viz. 2% malt extract with *p*H 6·8, was used throughout, and recent methods of estimation of enzymic activities were adopted.

3. The activity of both intracellular and extracellular enzymes was studied at three different stages, viz. (*a*) young vegetative stage, (*b*) old

mycelial (about-to-fruit) stage, and (*c*) fruiting stage. Side by side, intracellular enzymes of fruit-bodies collected from nature were estimated. In almost all cases the activity was studied both qualitatively and quantitatively.

4. The amount of extracellular enzymes has been found in all cases to be much larger than the corresponding intracellular ones according to the methods adopted by us. It therefore appears that the major portion of the enzymes formed in the cell is secreted externally into the medium to convert the food materials into an available form.

5. In all cases except that of catalase it has been found that the activity of enzymes in the vegetative state is greater than that in the fruiting or about-to-fruit stage. In the case of extracellular enzymes, where the total quantity of enzymes secreted is collected in the medium externally and taken into account, this statement is made with reservation, in view of the fact that a certain time elapses between the collection at the vegetative stage and at the fruiting stage, though they were inoculated at the same time. During this interval the activity of the extracellular enzymes might decrease, but this question does not arise in the case of intracellular enzymes.

6. Among the carbohydrases, the following were found to be present, viz. invertase, raffinase, maltase, amylase, emulsin, hemicellulase, cellulase, pectinase and ligninase, while lactase and zymase were absent.

7. Lipolytic and proteolytic enzymes were also found in small quantities.

8. Catalase was found in all cases as intracellular enzyme, and laccase was present in *Polystictus sanguineus*, *Daedalea flavida* and *Trametes lactinea*.

9. Of the *Polypores* studied, *Polyporus ostreiformis* was found to be the most active. It has been recently reported by Sharples (1928) and Bose (1934) as a suspected wound parasite on areca nut palms in Malaya and Calcutta respectively.

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Neoplasia following the Application of X-Rays to Inflammatory Lesions

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[Plates 7, 8]

It is of great importance, for scientific reasons and for the more limited purpose of safeguarding against possible dangers in X-ray therapy, to discover whether there may be conditions which facilitate the production of tumours by X-rays. Some light has already been thrown on this problem. Lacassagne and Vinzent (1929) (1933) caused inflammation in the groins of rabbits by the subcutaneous injection of *Streptococcus caviae*. The lesions in some of these rabbits were submitted to X-radiation, the dose in each instance being about 600 r. Such doses had a beneficial effect on the infective lesions, but sarcomas developed in five of the twelve survivors at the sites of irradiation. In a later experiment non-infected inflammatory lesions, produced in rabbits by the subcutaneous injection of sterile diatomaceous earth, were exposed to X-rays, the dose being 600 r. as before. Two of these rabbits, at the end of 22 and 34 months respectively, were dead with malignant tumours at the site of irradiation (Lacassagne 1933). Similar doses of X-rays given to rabbits in the absence of inflammatory lesions did not induce tumour formation (Lacassagne 1936). Since a total of 600 r. is well within the limit of dosage used for therapeutic purposes in man, it seemed desirable to carry out further experiments of this nature.

Experimental Technique

Non-infective foci of inflammation were caused in twelve young adult rabbits as follows: 3 c.c. of olive oil containing 10 % of finely powdered silica were injected subcutaneously in the right groins. Four days later, as no distinct swellings were present, the injections were repeated, 2 % of oleic acid having been added to the olive oil. On the following day 7 c.c. of a suspension of kaolin (10 % in water) were injected into the same sites. Three days later obvious swellings were present. Each rabbit was anaesthetized with "Evipan" and the swollen tissues were exposed to a single dose of 600 r., the rest of the rabbit being protected by a shield of lead. The conditions of irradiation were—250 kV, 4 mA, filter 0·5 mm. Cu, time approximately 23 min.

Results

One of the rabbits died from an overdose of the anaesthetic. After treatment the rabbits were kept in the country. There were two early deaths. Among the remaining nine, six have died with tumours at the sites of irradiation and three are still alive and free from growths (see Table I). Particulars of the six rabbits in which tumours developed are given below.

Rabbit 1. X-rays applied in November 1933. In September 1935 copious bleeding occurred from the right groin where a hard ulcerated swelling, 31 × 39 mm. in diameter, was present. Under anaesthesia a portion of the tumour was removed and an autograft inserted in the left groin, twelve young rabbits receiving grafts at the same time. The rabbit died 19 days later.

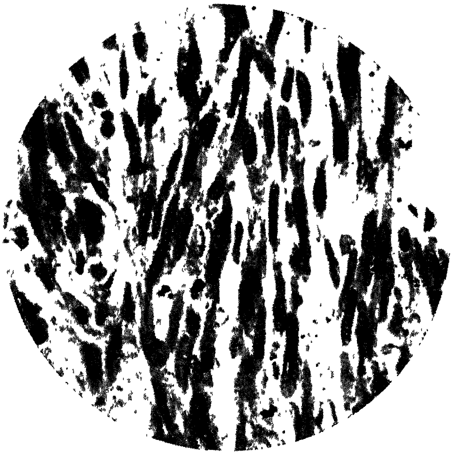

FIG. 1

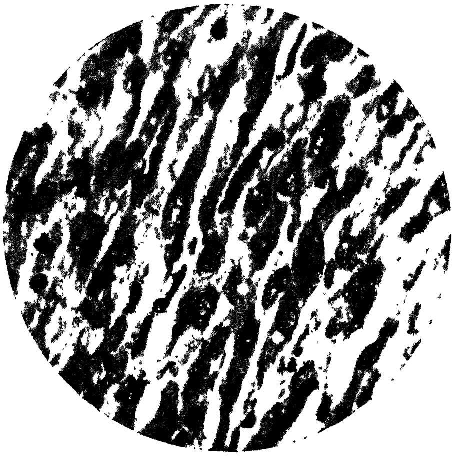

FIG. 2

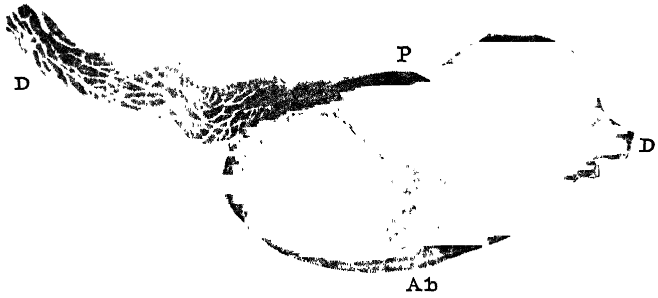

FIG. 3

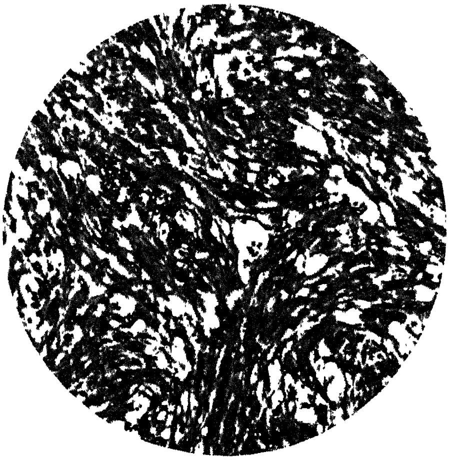

FIG. 4

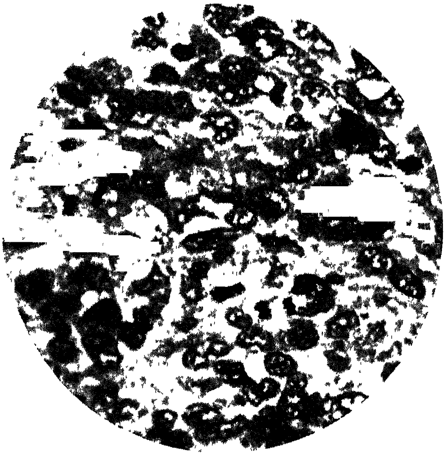

Fig. 5

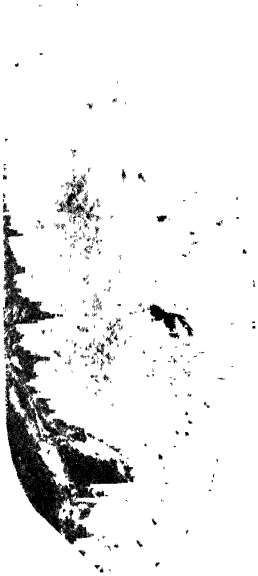

Fig. 6

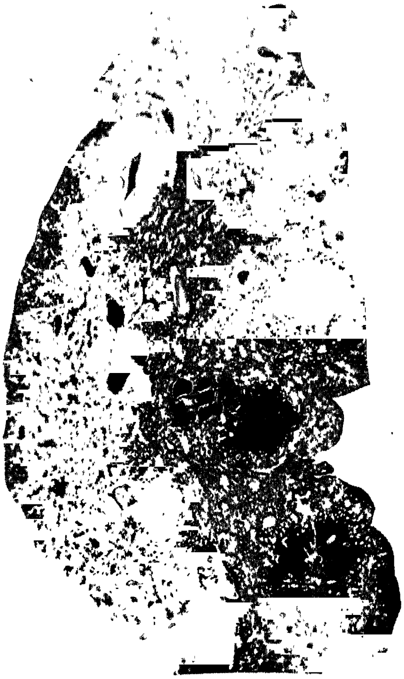

Fig. 7

P.M. The tumour is necrotic and foul smelling. Microscopic sections show it to be a spindle-celled tumour invading the subjacent fascia and muscle (fig. 1, Plate 7). There is no metastasis. The autograft has survived, but none of the heterografts has produced a tumour.

TABLE I

	Weight of rabbit when irradiated g.	Survival period months	Tumour at site of radiation	Metastasis	Nature of tumours
1	1500	22	+	−	Spindle-celled invasive tumour
2	1670	23	+	−	Not determined
3	1750	35	+	+	Spindle-celled sarcoma
4	1700	38	+	+	Spindle-celled sarcoma
5	1800	39	+	+	Polymorphic-celled sarcoma
6	1800	38	+	+	Spindle-celled sarcoma
7	1750	Still alive	−		
8	1600	Still alive	−		
9	1650	Still alive	−		

Rabbit 2. Found dead 23 months after irradiation.

P.M. Pneumonia appears to have been the cause of death. There is a hard swelling at the site of irradiation, measuring 21 × 16 mm. No metastases are seen. No satisfactory histological material is available.

Rabbit 3. Killed 35 months after irradiation.

P.M. A tumour is present in the right groin measuring 61 × 55 × 50 mm. It consists of a necrotic core surrounded by pale translucent tissue. Metastasis has occurred in the retroperitoneal, mediastinal and cervical lymph glands, and also in the lungs, liver, kidneys, diaphragm, skin and other organs. The tumours are spindle-celled sarcomas (figs. 2, 3, Plate 7).

Rabbit 4. Thirty-six months after irradiation the rabbit was sick and thin, and a hard, fixed irregular tumour was present in the right groin. A piece was removed and autografts were implanted in the left groin and axilla. Two months later the rabbit was killed.

P.M. In the right groin is an ulcerated tumour 60 × 50 mm. in size. Metastatic growths are present in the retroperitoneal lymph glands, obstructing the right ureter and causing hydronephrosis. There is a metastatic tumour also in the subcutaneous tissue of the right flank adjacent to the collateral vein which connects the femoral and axillary vessels. The autograft in the left groin has survived. Sections show the tumours to be spindle-celled sarcomas (fig. 4, Plate 7).

Rabbit 5. Thirty-six months after irradiation a hard, flattened, ulcerated tumour measuring 17 × 21 mm. was noticed in the right groin. Under anaesthesia a portion was removed and a graft was inserted in the left groin. Three months later the original tumour measured 58 × 47 mm. in its accessible diameters. A second tumour was present in the back of the right thigh. The rabbit appeared ill and was killed.

P.M. The tumour in the groin is distinct from that in the thigh. The latter is encapsulated except at one place toward the anus where the skin is involved in the growth. The dimensions of this tumour are 77 × 63 × 55 mm. The only metastases are in the retroperitoneal lymph glands at the bifurcation of the aorta. Both the primary tumours and the metastases are polymorphic-celled sarcomas. Many of the cells have very large nuclei, and in places there are aggregations of nuclei. Numerous mitoses are seen (fig. 5, Plate 8).

Rabbit 6. Thirty-eight months after irradiation the rabbit was ill and emaciated, and a hard swelling was present in the right groin with ulceration of the overlying skin. The rabbit was killed.

P.M. The primary tumour is largely necrotic and measures 31 × 24 × 24 mm. There are widely distributed metastases in the lymph glands, lung, liver, kidneys, adrenals, and other organs (figs. 6, 7, Plate 8). The tumours are spindle-celled sarcomas.

Discussion

The suggestion may be made that the tumours in our rabbits were the consequence of inflammation apart from irradiation. In man it is recognized that a chronically inflamed tissue after a long lapse of time may become the seat of cancer, and in the past inflammation was regarded as a cause of cancer. Experiments have consistently failed to support such a view, and it seems probable that the role played by chronic inflammation in carcinogenesis, apart from the action of X-rays, is to bring about a concentration and retention in the affected tissues of the specific agents of cancer, or in some other way to facilitate neoplastic responses to specific agents. A more extensive set of experiments is now being undertaken and will include appropriate controls. Meanwhile, attention may be drawn to the fact that, with the exception of two rabbits which died early and two (Nos. 1 and 2 of Table I) in which the nature of the tumours remains somewhat uncertain, cancer has appeared in the groins of four of seven rabbits following the application of X-rays in a single dose of 600 r. to these regions while in a state of inflammation. Experience suggests that broadly speaking the etiology of cancer in man and in rabbits is comparable, with the proviso that the period intervening between cause and effect is longer in man. Moreover, clinical evidence indicates that for the production of cancer in man by exposure to tar, shale oil, arsenic, ultra-violet light, or X-radiation, an interval of some 10 years or more is usually required, a period beyond the rabbit's average limit of life. This long intervening period between cause and effect might possibly obscure the origin of cancer in certain instances.

We wish to express our thanks to the British Empire Cancer Campaign for financial assistance, to the Royal College of Surgeons of England who kindly made provision for the care of our rabbits at the Buckston Browne Research Farm, and to Professor J. M. Woodburn Morison for his interest and encouragement.

SUMMARY

A focus of inflammation was caused in the groin of each of twelve rabbits by injections of kaolin and of finely powdered silica suspended in olive oil. These foci were exposed to X-rays, each receiving a single dose of 600 r. Among nine rabbits thus treated and surviving for 2 years or longer tumours appeared in the irradiated tissues in six. In four of these instances the tumours were sarcomata which had produced metastases.

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DESCRIPTION OF PLATES

Plate 7

FIG. 1—Primary tumour, rabbit 1. × 375.
FIG. 2—Primary tumour, rabbit 3. × 375.
FIG. 3—Metastatic tumour perforating diaphragm, *D*; *P*, pleural cavity; *Ab*, abdominal cavity. Rabbit 3. × 5.
FIG. 4—Metastatic tumour compressing ureter, rabbit 4. × 135.

Plate 8

FIG. 5—Primary tumour, rabbit 5. × 375.
FIG. 6—Kidney showing metastases, rabbit 6. × 8.
FIG. 7—Lung showing numerous metastases, rabbit 6. × 8.

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Experiments on Muscle Haemoglobin *in vivo*; the Instantaneous Measurement of Muscle Metabolism

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(*Communicated by Sir Joseph Barcroft, F.R.S.—Received* 31 *March* 1937)

[Plates 9–11]

Introduction

Muscle haemoglobin is fitted to act both as a short-time oxygen store and as an intracellular indicator of oxygen tension, with an "indicating time" of less than one-hundredth of a second (R. Hill 1936; Millikan 1936). We can now measure its rate of oxygenation and reduction in a normal muscle, during rest and contraction, both with unimpaired and arrested circulation, and are thus enabled:

(1) to obtain direct evidence about the function of muscle haemoglobin in the living tissue;

(2) to measure the instantaneous oxygen consumption before, during, and after contraction;

(3) to determine the minimum intracellular oxygen tension at which normal muscle can operate; hence the substrate-enzyme affinity of the "limiting" oxidation enzyme; and

(4) from the rate of oxygen supply to follow changes in the rate of blood flow during and after muscular contraction.

The present paper describes how the saturation changes in the living, unexcised muscle can be followed photoelectrically and recorded automatically. It also deals with the results which have so far been obtained on the soleus muscle of the cat, and their bearing on topics (1), (2), and (4). Topic (3) demands a number of special assumptions and will be reserved for a later communication.

The method to be described provides us in effect with an intracellular "metabolic microscope" with instant action, which is distinguished from previous instruments for measuring metabolism in being both chemically specific and time sensitive. All manometric and gas-exchange methods are subject to a delay of several seconds, at the very least, owing to the slow rate of diffusion from the interior of the cell to the point of measurement, while chemical analysis of tissue pulps and extracts are generally subject to even greater time uncertainties. The previously available time-sensitive lines of approach, on the other hand, such as the measurement of electrical, thermal, volumetric, morphological, or opacity changes, are integrative in their action, depending upon the sum of the effects of the separate chemical reactions involved. The use of muscle haemoglobin as an indicator possesses advantages of both groups of methods; it occurs naturally in the liquid phase in the interior of the muscle cell, responds without appreciable time lag, and, owing to its very small Bohr effect, is little affected by *p*H changes. It combines with only oxygen and carbon monoxide, but since the latter was excluded from our experiments, it may here be regarded as being specific for oxygen alone. Finally, the ready reversibility of its link with oxygen assures us that its use as an indicator is quite independent of the manner in which oxygen is being utilized inside the cell.

I—METHOD

When a muscle contracts it may undergo a number of optical changes in addition to the alteration in the saturation of its muscle haemoglobin, which we wish to measure:

(1) it becomes shorter and thicker, thus altering the path of a beam of light sent through it;

(2) its blood content may change due to the constriction or dilatation of its capillaries, while the degree of saturation of this blood haemoglobin may also change;

(3) the muscle becomes less opaque, probably due to changes in the lactic acid and phosphagen concentration (von Muralt 1934).

Our experimental problem is to discover a red muscle which is suitable for optical observations, involving the least possible interference with normal physiological activity, and to devise experimental conditions which will minimize the adverse effects of these three factors.*

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* The study of tissue metabolism by means of optical indicators inside the cell is not new. The "time of reduction" of cytochrome as observed visually in a spectroscope was used by Keilin (1925) as an indication of metabolic activity, and Stier (1935) has made a careful quantitative study of this property in yeast. Haas (1934) applied a very sensitive photoelectric cell technique to the same problem. Von Muralt (1934) has correlated total opacity changes of frog's sartorius muscle, which he measured photoelectrically, with phosphagen and lactic acid content. The present method differs from any of these in using an indicator which is known to combine reversibly with the indicated substance in a very short period of time; it differs also in utilizing a two-colour photocell measuring instrument, which ignores, in a large measure, irrelevant optical changes taking place in the tissue.

Preparation

The soleus muscle of the cat has been chosen, because it contains ample muscle haemoglobin, a relatively small amount of blood (see below), is of an ideal thickness for optical work (2–4 mm.), and is so situated that it can very easily be laid bare for insertion in a special optical trough, without serious stretching and without interfering either with the blood supply or the nerve supply. The preparation requires a very small amount of dissection, and hardly any disturbance to the other parts of the leg.

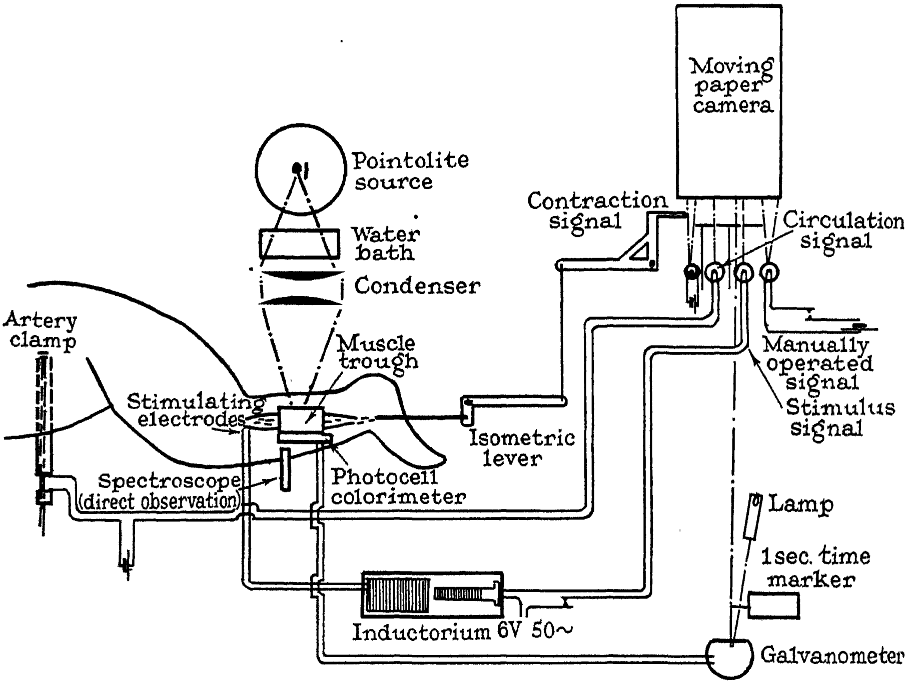

Fig. 1—Diagram of the experimental set-up. The camera records: (1) galvanometer deflexions: degree of oxygenation of Mgb. (2) mechanical contraction of the muscle (isometric lever). (3) electrical stimulation (nerve or direct on muscle). (4) artery clamp, whether open or shut. (5) manually operated signal for registering visual appearance of spectroscopic bands, etc. (6) 1 sec. time marker (interrupting galvanometer beam).

The Experimental Arrangement

There are three component parts of the apparatus:

(1) the optical system, including the muscle trough,
(2) the photocell colorimeter,
(3) the recording system.

The general arrangement of the whole set-up can be seen from fig. 1, while details of its components are given in figs. 2, 3, and 4.

1—*The Optical System* (fig. 2).

Light from a 1000 c.p. pointolite lamp passes through a heat filter, consisting of a trough 2 in. thick containing a 10 % $CuSO_4$ solution, and is focussed by means of a 6 in. condenser lens on the muscle holder, where it suffers a right-angle reflexion from a totally reflecting prism, passes through the muscle, and either into a direct-vision spectroscope for visual observation or into a photocell colorimeter for recording. The muscle holder or trough (fig. 3 *a*) has passed through several stages of development before arriving at its present fairly satisfactory form.

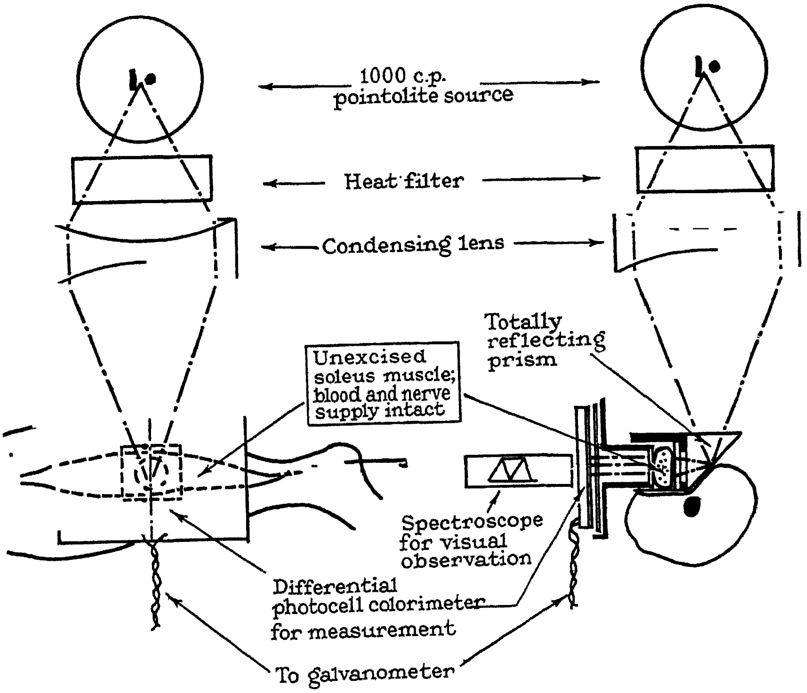
1000 c.p. pointolite source
Heat filter
Condensing lens
Totally reflecting prism
Unexcised soleus muscle; blood and nerve supply intact
Spectroscope for visual observation
Differential photocell colorimeter for measurement
To galvanometer

FIG. 2—The optical system.

Its chief features are:

(*a*) The glass plates, by means of which the muscle is confined, are kept parallel by means of a simple link and lever mechanism. This insures a nearly uniform light path (fig. 3*a*).

(*b*) Instant release and adjustment to any desired trough thickness is achieved by means of the lever system and calibrated scale. Once in position the trough thickness remains unchanged. The muscle is always clamped just tightly enough to ensure its touching the glass plates on each side, and loosely enough to prevent clamping off the circulation.

(*c*) The trough is so designed that the muscle when in it is displaced a minimum distance from its normal position in the leg. The reflecting prism and the very thin bottom member of the muscle holder were designed with this end in view.

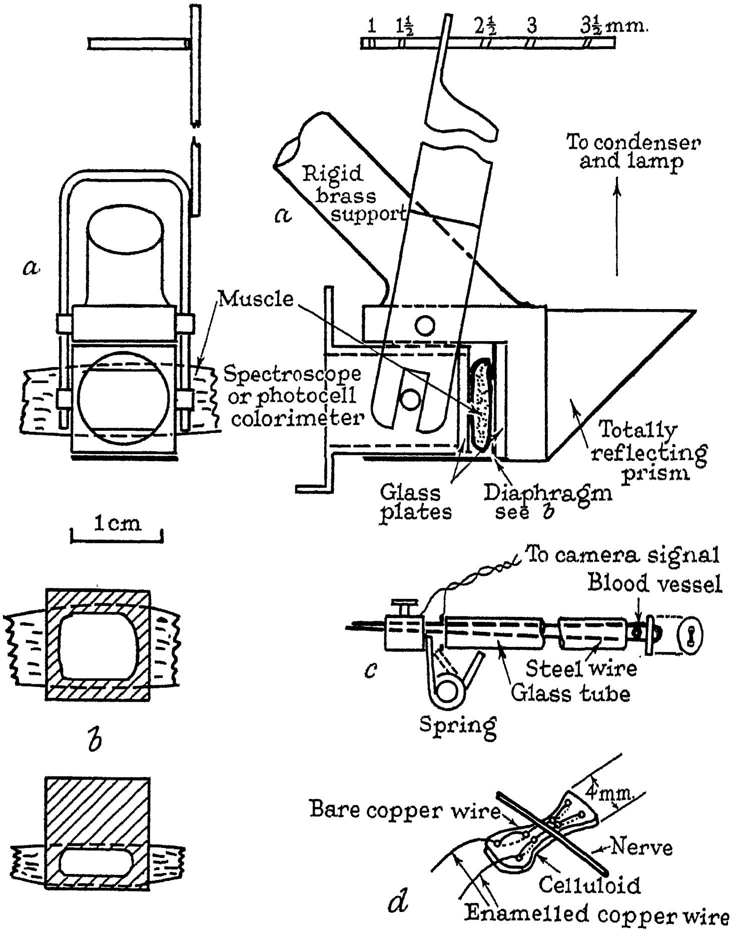

FIG. 3—Details of special apparatus. *a*, the optical trough for the muscle, insuring parallelism and allowing instant adjustment of thickness. *b*, different stops to be inserted into the muscle trough for muscles of different thickness. *c*, artery clamp; electrical connexion to signal lamp. *d*, stimulating electrode to be slipped under nerve.

(*d*) A number of diaphragms or stops (fig. 3*b*) are available to vary the area being illuminated according to the size of the muscle.

(*e*) The trough is brazed directly on to a $\frac{3}{8}$ in. brass supporting rod, which is firmly clamped to the table. This ensures rigidity of the whole system, which is most important, for any distortion of the optical system during contraction, throwing either more or less light on any part of the photocell system, would be fatal for the accuracy of the measurements. This source of error can fortunately be controlled by the cyanide method described below.

2—*The Differential Photocell Colorimeter* (fig. 4).

This is fastened directly to the back of the muscle trough. Its principle is as follows: at the red end of the spectrum reduced haemoglobin (either muscle or blood) absorbs more light than the oxygenated pigment, whereas

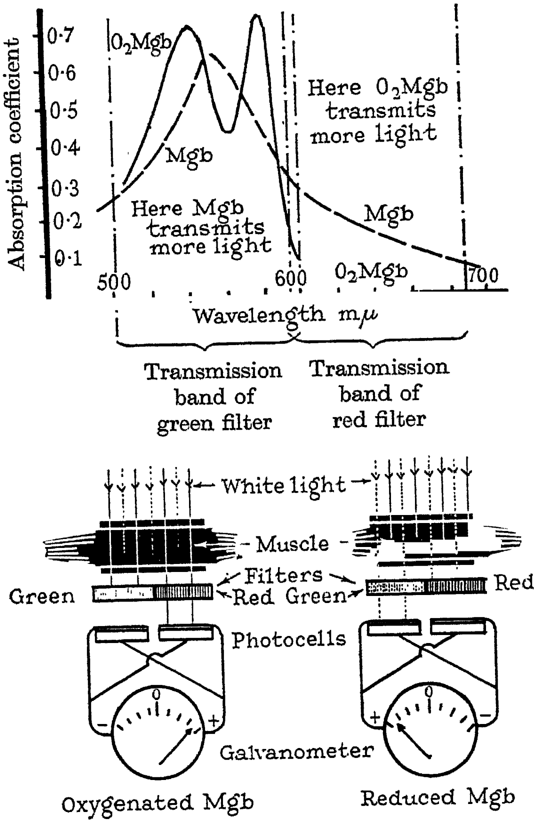

Fig. 4—The principle of the "spectral balance" photocell colorimeter as applied to muscle haemoglobin *in vivo*. Data for absorption coefficients from Theorell (1934*a*).

in the green and yellow, the region of the α and β bands, the position is reversed, the "oxy" form absorbing more light than the reduced. By balancing the one spectral region against the other, using two-colour filters and selenium photocells connected in opposition to each other, oxygenation of the pigment can actually be made to reverse the direction of the galvanometer current. The scheme possesses the enormous advantage for *in vivo* experiments of being nearly—but not quite—independent of fluctuations in the light source and in the total opacity of the muscle. This independence is complete at the intermediate null point at which no galvanometer current flows, and its degree of failure at other degrees of saturation can be calculated; it can also be controlled by the cyanide method (see below). It has been shown elsewhere (Millikan 1933) that a linear calibration curve, to within 1 %, is obtained if the *change* in light absorption caused by oxygenation is less than about 4 % of the total transmitted light, and this condition has been fulfilled in practice throughout the present experiments.

The colour filters are mounted side by side in front of the two photocells, which are in practice a single "S" type "Electrocell", which has been cut in two with a hack saw. Suitable filters for muscle haemoglobin are Wratten No. 25 (red), and Wratten No. 56 (green). A low-sensitivity galvanometer of fairly rapid period is used: Pye, 10^{-8} amp./mm.; resistance, 500 ohms; period, 1·2 sec.

Filters and photocells are mounted as a single unit in an ebonite frame; a simple screw adjustment enables the filter-photocell unit to be fixed in that position where the galvanometer deflexions are about equidistant from the zero point on each side. The unit can also be slipped aside for the direct spectroscopic observation of the bands, so that one can alternate visual with photoelectric observation.

3—*The Recording System.*

This consists of a moving paper camera (12 cm. bromide paper) fitted with a number of signal lamps so that records may be made not only of the galvanometer deflexions, but also of the degree of contraction of the muscle (shadow cast by isometric lever), the condition of the arterial supply (whether clamped off or unrestricted), and the time of stimulation (figs. 1 and 3*c*). In the experiments reported in this paper, the bromide paper moved at the rate of 1–2 mm./sec., and the recording time for oxygenation was approximately 1 sec., which was the period of the galvanometer. The "time of response", i.e. the latent period for a quick change to be indicated, is of the order of $\frac{1}{5}$ sec.

Procedure of an Experiment

Normal cats were used, preferably rather small ones (2–2·5 kg.); they were anaesthetized with dial, injected intraperitoneally. The artery clamp was then fixed in place, in the earlier experiments on the femoral artery, but in the later ones, always on the descending aorta, a short distance below the kidneys.

The muscle was next laid free, and the stimulating electrodes placed on the muscle or nerve, the inductorium being so adjusted to give a maximal contraction in either case. All stimuli were 50 c./sec. The muscle was then placed in the muscle trough, and a stout wire connected to the isometric lever was thrust through the Achilles tendon. All exposed parts were covered with cotton-wool soaked in warm saline, the whole preparation being kept warm by means of an electric heater. No very accurate temperature control was maintained; for reliable temperature-metabolism experiments this factor will have to be more carefully regulated.

Notation and Calculation

We shall be concerned in this paper chiefly with "rates of change" of oxygen saturation in the muscle. But since the final values are usually approached asymptotically, this rate of change becomes constantly less as the process goes on. For the sake of simplicity, we shall therefore deal here only with "initial rate of change", drawing a tangent to the curve during the first second or two of change, and using the slope of this line as our criterion for "oxygen consumption", "recovery", etc. Since relative values are our principal concern, it is usually convenient to express this slope in terms of "percentage of total Mgb capacity per second" (% Mgb/sec.). When absolute values for the oxygen consumption are required, we must multiply these values by the total oxygen capacity of the Mgb contained in 1 g. of muscle. For the cat soleus, the Mgb content averages about $\frac{1}{2}\,\% = 6{\cdot}6$ cu. mm. O_2/g. muscle. In very red muscles this value may be doubled.

Controls

1—*The Relative Amounts of* Mgb *and* Hb *in the Living Muscle: Band Positions.*

Our evidence concerning the relative contributions of the blood and muscle pigments to the total light absorption depends upon the fact that the α absorption band of muscle oxyhaemoglobin is 3–4 mμ farther to the red than that of blood oxyhaemoglobin. The quantitative estimation of the two pigments depends upon the following observations, most of which have been first reported by other investigators, but all of which have been repeatedly confirmed in the course of the present work:

(*a*) The band position as measured with the reversion spectroscope is not affected by the optical properties of the medium. Thus Ray and Paff (1930) found that the band position of intact red muscles was the same as that of their clear watery extract. We have confirmed this general result in another way by comparing the band position of haemoglobin in water and in a heavily dispersing medium, milk. To avoid glare in the spectroscope, the intensity of the light passing through the clear solution was first reduced many times by interposing a sheet of filter paper in the light beam. In both cases, the Hb concentration was 0·5 %, the trough thickness 1 mm. and only the blood pigment was used. The following results each represent an average of twenty readings:

Clear Hb solution ...	5763 A ± 3
Hb in milk	5766 A ± 3

The difference between the two sets is seen to fall within the experimental uncertainty of the determinations.

(*b*) The relative amounts of Mgb and Hb in a mixture is linearly related to the band position. The following table shows the fraction of Mgb in a known pigment mixture, as calculated from the band position, assuming the linear relation to hold:

True fraction $\frac{\text{Mgb}}{\text{Mgb}+\text{Hb}}$	Fraction of Mgb calculated from band position, assuming linearity	
	Watson (1935)	New*
0	—	—
0·25	0·20	0·21
0·50	0·48	0·43
0·75	0·77	0·75
1·00	—	—

* I am much indebted to Dr. Carl Baumann for making these determinations and for many other aids in the earlier stages of this work.

The band position is thus seen to be a reliable criterion of the composition to within about 5 %; the error appears to be somewhat less than this at the "all Mgb" end, which is the important one for our experiments.

(*c*) The band position in the normal living cat soleus muscle with circulation intact corresponds almost exactly with that of Mgb alone. This is shown in the following table, in which each figure is the average of 5, 10 or 20 individual spectroscope measurements:

	Band position			
	Lowest reading	Highest reading	Average	No. of animals
Cat blood	5759	5773	5766	9
Crystal Mgb (horse heart)	5806	5812	5809	3
Crystal Mgb (horse heart) Watson (1935)	—	—	5808	Many
Living cat soleus	5803	5819	5810	12
Mgb/total Hb in cat soleus	0·84	1·23	1·02	12

2—*Cyanide Control.*

It is most important that we eliminate the possibility of galvanometer deflexions which are due to incidental changes when the muscle contracts or relaxes, such as mechanical distortion of the optical system, the movement of a dark spot from in front of one photocell to the other, changes in the blood content of the muscle, etc. All these possible sources of error can happily be controlled, and controlled quantitatively, at one time, by the simple expedient of injecting a small amount of KCN into the tissue (1/2 c.c. of 1 % solution). This poisons the oxygen uptake of the cell, so that Mgb remains constantly saturated, but the muscle can still contract anaerobically for a time, and the blood supply is but slightly affected, if at all. The galvanometer deflexions, when the muscle is stimulated, or when the circulation is cut off, are now simply a measure of these *errors*, the spectroscopic change which we are normally trying to measure being now completely eliminated. Such a control usually concludes every experiment; the normal "wobble" in the base line is from 5 to 15 %, and averages about 10 %. The record of a typical cyanide control is given in Plate 11.

3—*Direct Spectroscopic Observation.*

The small direct-vision spectroscope which can replace the photocell colorimeter at any instant gives a most valuable check on the general appearance of the bands at any time, which can be correlated with the galvanometer position; the band appearance, however, can neither be quantitatively described, nor can it be conveniently recorded during periods of rapid fluctuation; this control must, therefore, remain only crudely qualitative.

4—*Control Still Lacking.*

If the oxygen affinity of the Mgb were to change suddenly during the contraction itself, due, let us say, to reorientation of surface forces inside the muscle fibre, the rate of disoxygenation of the pigment would not measure accurately the oxygen uptake. Such a change could probably be detected experimentally by comparing a "dissociation curve *in vivo*" of the Mgb in a relaxed muscle with one in a state of contraction. Varying oxygen tensions would be provided by a series of perfusion liquids, the oxygen saturations being measured by the present device, while complications caused by oxygen utilization could be avoided by putting a little cyanide in the perfusate. Such experiments are now being planned.

In the absence of such proof, we may, however, mention the following facts which strongly *suggest* that if a change of affinity does take place, the change is not a very large one:

(*a*) The KCN control shows that contraction does not lower the affinity enough to cause a sensible dissociation at the relatively low oxygen tension of the cyanized muscle. A big increase in affinity is unlikely because Mgb gives up its oxygen so fast when the muscle does contract.

(*b*) The kinetics of Mgb in solution are not much affected by the presence of considerable amounts of protein (Millikan 1936).

(*c*) Theorell (1934*b*) found that the affinity of Mgb was not very much affected by *p*H.

(*d*) The ease with which Mgb is extracted suggests that it is not very firmly fastened to any of the more solid parts of the muscle fibre.

II—EXPERIMENTAL RESULTS

A—*Resting Metabolism and Recovery Metabolism*

The record obtained in a typical experiment has been reproduced in Plates 9 and 10.* It opens with the muscle resting, and its circulation unimpaired. The Mgb is saturated with oxygen. At *a* (again later at *h′*, *l′* and *e″*) the aorta is clamped, the ensuing gradual deoxygenation of the Mgb, *a* to *b*, measuring the resting oxygen metabolism, about 1 % Mgb/sec. The irregularity from *b* to *c* may be disregarded, for during this period the photocell colorimeter is disconnected so that direct visual observation of the muscle haemoglobin bands may be made; they are reported as "faint". This part of the record has been cut off to save space. The aorta clamp is released at *d*, the Mgb becoming reoxygenated at a rate which measures the effective circulation; it is seen to proceed at a rate of 4·1 % Mgb/sec.

B—*Oxygen Consumption during Contraction*

If the muscle is made to contract during such a period of arrested circulation, as at *o′*; or if the stimulus and the artery clamp are applied simultaneously, as at *m*, *t*, *y*, and *q″*, the Mgb becomes disoxygenated at a very much faster rate (average about 14 %/sec.), indicating a correspondingly increased oxygen consumption. At the end of the stimulus, the Mgb is unable to replenish its oxygen store: *n*, *p*; *p′*, *r′*..., until the circulation has been restored: *s*; *s″*. There is, it is true, a certain recovery visible in the reproduced record, but its amount varies from experiment to experiment, and it is never greater than the uncertainties of the method as measured by the KCN control. It is here about 10 % of the total Mgb capacity, but is usually about half that amount, and may be considered principally as an artefact.

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* The record of a single experiment is given practically in its entirety in preference to a number of carefully selected fragments from several experiments, partly because all the relevant points are here illustrated, but principally because this treatment conveys some idea of the general reproduceability and steadiness of the method. The reported conclusions, however, as well as the numerical averages given in the text, have all been reached from the study of some twenty-five similar records made from as many different animals.

C—*Contraction with Unimpaired Circulation; the Role of Muscle Haemoglobin*

We can follow what happens to the *normal* muscle during contraction by giving the stimulus by itself without interfering with the circulation. This can be seen at *e*, *g*, etc.; *t*′, *v*′, . . . ; *g*″, *i*″, ... The rapid rate of disoxygenation—which averages about 9 %/sec. in the present record, and has reached as much as 25 %/sec. in some experiments—provides the most satisfactory evidence which we now possess in support of the theory that muscle haemoglobin acts principally as a short-period oxygen store, thus providing an "oxygen buffer" in a system whose intermittent and extreme activity tends to produce correspondingly large fluctuations in the rate of oxygen flow from the blood to the final oxidation enzymes. Mgb helps to tide the muscle over from one contraction to the next by smoothing out these fluctuations.

It is interesting to note that the distribution of Mgb in mammalian musculature furnishes confirmatory evidence for this conclusion: red muscles, which contain much Mgb, are on the whole more slow acting than white ones, and they are more capable of sustained activity. A high concentration of Mgb is also found in that organ, which can least afford either to rest or to contract an oxygen debt, namely, the mammalian heart. (For a full discussion of red and white muscle, see Needham 1926.)

D—*Recovery Rate after Short Tetanus*

A striking feature of the present findings is the very rapid recovery of the muscle after a maximal tetanic contraction of a few seconds duration, suggesting that a very large fraction of the total oxygen required by normal muscular activity is used up at the moment of contraction and not afterward. An example of this quick recovery may be seen from *e*″ to *o*″ on Plate 10, where within 10 sec. after the end of a series of contractions, the oxygen consumption is down to 140 % of the resting rate, whereas during the contraction it rose to more than 450 % of the resting rate. The numerical values measured on Plate 10 are as follows:

e″: resting metabolism: 1·6 % Mgb/sec.

m″: maximal contraction: 7·1 % Mgb/sec. 7·1/1·6 = 450 % of resting rate.

n″: release from contraction.

o″: circulation clamp applied 7 sec. after *n*″; ensuing oxygen consumption: 2·2 % Mgb/sec. 2·2/1·6 = 140 % of resting rate.

Since the circulation was not clamped off during contraction *m*″, the slope of the curve represents only a part of the oxygen actually consumed during that period. The true total rate is probably about twice the figure here given, i.e. about 900 % of the resting value.

III—DISCUSSION

A—*Intermittent Supply or Intermittent Demand? Does the Blood Flow Stop During Contraction?*

There is now strong evidence that muscle haemoglobin acts in repetitively contracting muscle as an oxygen "buffer" or store to tide the muscle over from one contraction to the next. There are two different ways in which it might do this. There might either be large fluctuations in the *supply* of oxygen caused by constriction of the blood vessels during contraction; or there might be large and sudden changes in the *demand* for oxygen during contraction, which the normal blood supply could not deal with. The first alternative leads us to the much-debated question of the blood flow through a muscle during contraction, while the second presents us with the fundamental problem of intracellular chemical kinetics of contracting muscle. It is therefore worth while to weigh carefully the merits of the two alternatives, and to attempt to assess their relative importance in normal activity of red muscle.

First let us consider the evidence of the present experiments. The expected changes of oxygenation of Mgb according to the theories of Intermittent Supply and Intermittent Demand are graphically presented in fig. 5, which was drawn up on the basis of the following extreme assumptions:

(*a*) Intermittent supply, fig. 5*a*, cessation of flow during contraction; uniform oxygen demand rising gradually during continued intermittent stimulation from its "resting rate" to its final "active rate".

(*b*) Intermittent demand, fig. 5*b*; blood flow not stopped during contraction, gradually rising during period of activity; intermittent oxygen demand, and rapid oxygen utilization.*

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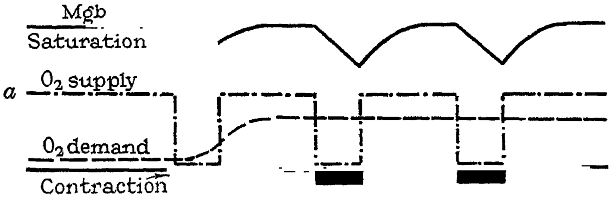
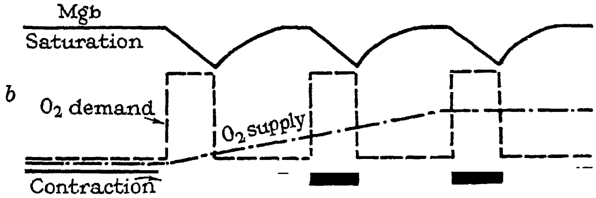
* The solid lines of fig. 5*a* and *b* represent the degree of oxygenation of the intramuscular Mgb, and their positions are determined by the differences between the rate of oxygen supply and the rate of oxygen utilization (see fig. 5*c*). A constant known saturation tells us nothing about the absolute values of these two quantities but only the fact that they are equal. We can, however, deduce their individual values from the *changes* in saturation which occur when we manipulate the two factors under our control, namely the state of contraction of the muscle and the state of the systemic circulation.

The two alternatives are not mutually exclusive, and our question must therefore not take the form, "Which is right?", but rather "How large a part does each play?"



Intermittent supply



Intermittent demand

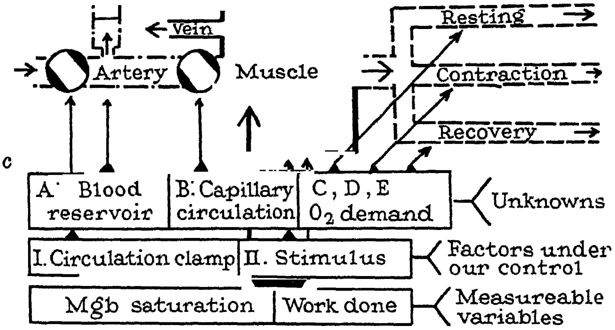


FIG. 5—Metabolism and muscle haemoglobin saturation. *a*, changes in Mgb saturation under conditions of intermittent supply. *b*, changes in Mgb saturation under conditions of intermittent demand. *c*, diagram of oxygen supply, storage, and utilization in muscle.

Evidence from Experiments with Normal Circulation.

It is seen that the essential difference between the solid lines of fig. 5*a* and *b* lies in the difference of behaviour between the first and succeeding contractions of a series. If the supply is intermittent, we should expect the initial dip to be shallower and less sudden than those subsequent to it, whereas on the intermittent demand theory the dips should all be more or less alike. In Plates 9 and 10 we have the actual records of three such series, viz. *e*, *g*, *i*, *k*, ..., *t'*, *v'*, *x'*, *z'*, *b''*, ..., *g''*, *i''*, *k''*, *m''*. In all three of these series, the first dip is indistinguishable from the successive ones (within experimental error), this conclusion supporting the intermittent demand theory.

Evidence from Experiments with Blocked Circulation.

If the blood flow in the muscle is stopped by a maximal contraction, we should expect the rate of disoxygenation of the Mgb to be the same, no matter whether the supplying artery was being clamped off at the same time or not. On the other hand, if muscular contraction *fails* to stop the blood flow, the application of the artery clamp along with the stimulus should increase the rate of deoxygenation. This has, in fact, been found to occur in every case; for example, compare Plate 9, *e*, unclamped, 8 %, with *m*, clamped, 14 %.

TABLE I—BLOOD FLOW THROUGH SOLEUS MUSCLE DURING CONTRACTION

The figures in cols. 2–7 are all given in % Mgb/sec.

1	2	3	4	5	6	7	8
	Disoxygenation during contraction			Normal oxygen supply			
Exp.	Clamped	Un-clamped	Difference = 2 − 3	Normal recovery rate	Resting uptake	Normal supply = 5 + 6	% change on contraction
e–s	14·3	8·9	5·4	4·3	1·0	5·3	+ 2
t–x	14·3	9·5	4·8	4·3	1·0	5·3	−10
n′–c″	13·3	9·6	3·7	3·9	1·4	5·3	−30
g″–s″	14·3	6·9	7·4	4·3	1·6	5·9	+26
						Average	− 3

But if the blood flow is not reduced to zero during maximal contraction, what is its value? Is it affected at all? A quantitative answer to this question is attempted in Table I using the data supplied by Plates 9 and 10. It must be admitted that the accuracy of the method for calculating relative blood flows is low, and it is not claimed that the figures have more than an order-of-magnitude significance; nevertheless, they do strongly suggest a "non-interference" behaviour, at least during the first few seconds of contraction. The table has been constructed as follows: The rates of deoxygenation during contraction with and without the arterial supply cut off are given in cols. 2 and 3, the difference between them in col. 4. This difference is regarded as a measure of the oxygen supply during contraction, and hence as an indication of the blood flow. The normal oxygen supply to the muscle, which is considered to indicate in the same way the normal blood flow, is obtained from the reoxygenation rate of the muscle at rest, col. 5. To obtain the total oxygen supply, col. 7, we must add that which is being used up as fast as it is supplied, namely the amount required for resting metabolism, col. 6. The ratio of col. 4 to col. 7 tells us the percentage increase or decrease in the blood flow brought about by contraction.

Direct Measurement of Blood Flow during Contraction; Experiments of Verzar, Rein, Anrep and Kramer.

The literature has become so copious and contradictory on this subject that it will be necessary to limit our discussion to those four experimenters and their colleagues whose work stands in closest relation to the present work, and merely to indicate rather than to detail the points of agreement and disagreement. Since all observers are agreed in finding a marked and often prolonged hyperaemia *after* contraction, we shall limit ourselves to what happens at the very start of a tetanic contraction, and during its course.

Verzar (1912) measured the blood flow and gas metabolism of the cat's gastrocnemius by tying a graduated 1 ml. pipette into the saphenous vein and deflecting the venous outflow into it by a bulldog clamp on the femoral vein after all the collateral blood vessels had been tied off. This required considerable mutilation of the preparation; it was found desirable "to cut away nearly all the adductor muscles". The blood flow into the pipette was necessarily slow, sometimes taking over a minute, so it was only possible to measure blood flows over fairly long time intervals. Usually the tetanus was continued until the pipette was full, and this time of filling was taken as the measure of average blood flow during the contraction. Verzar concludes from his measurements that "the rate of blood flow is decreased during muscular activity and increased after, as has been shown by earlier observers". The results were, however, admittedly variable, and of the four relevant protocols in his paper, two show increases and two decreases. It has been suggested that this variability may be due to stimulation at one time of vaso-constrictor and at another time of vaso-dilator fibres in the common nerve trunk.

Rein and his collaborators (Keller, Loeser and Rein 1930; Rein, Mertens and Schneider 1935) have measured the blood flow of dogs in the unopened femoral artery and femoral vein by means of Rein's thermostromuhr, using a high frequency stimulation (150–400 c./sec.) of the muscle itself. They failed to find a cessation of flow during tetanic contraction, but either normal blood flow or a gradual increase, following a momentary period of uncertainty at the very start of the contraction. To answer doubts as to whether the thermostromuhr can record cessation of flow, Rein, Mertens and Schneider (1935) have shown that though the contracting muscle is unable to block the circulation, an artificial clamp on the femoral artery does do so. Rein's experiments have been criticized on the ground not only of the alleged technical inadequacy of the apparatus to present a true picture of sudden changes of blood flow inside the blood vessel, but also on the ground that the thermal element may be measuring blood from other parts of the limb as well as from the stimulated muscle.

Anrep and his co-workers (1934, 1935, 1936) have also worked on the dog's gastrocnemius, which they have perfused at constant pressure with defibrinated or heparinized blood from the same animal, measuring both the arterial and venous flow by means of the hot wire anemometer, after they had carefully tied off all collateral blood vessels. In striking contrast to Rein, they have found practically complete cessation of flow during maximal tetanic stimulus, which they take to be due to compression of the intramuscular vessels. Rein has suggested that the low frequency stimulus, which Anrep used, stimulated not only the striated muscle, but also the specific vaso-constrictor fibres in it. If this criticism is valid, it introduces one more anomaly into an already confused picture, for the present experiments agree essentially with those of Rein, though they, like Anrep's, involved low frequency stimulation (50 c./sec.) of both motor nerve and of the muscle itself.

Kramer (1936*a*) has applied his photoelectric technique on unopened blood vessels to the problem of determining linear rates of flow. Two photocells placed at two different points on the femoral artery measure the time it takes "colour waves" to pass from one to the other. These colour waves consist of normal respiratory fluctuations in arterial saturation. The entire musculature of the hind limb was stimulated (500 c./sec.). Kramer's findings confirm those of Rein and stand in contradiction to those of Anrep, the linear rates of flow rising gradually during the tetanic contraction to a final value about 65 % above the normal value. This slow increase is not in any way incompatible with our findings of "no essential change", inasmuch as our calculations were made from "initial slopes", i.e. from the first two or three seconds of the contraction.

In assessing the validity of the present method as a means of measuring blood flow, as compared with those referred to above, the following balance sheet of its credits and liabilities may be of some value:

In its favour:

(1) The present method is the only one which measures the desired

phenomenon *in the organ* in which it is taking place, and *at the time* when it is taking place.

(2) It requires no interruption or disturbance either with the blood or nerve supply of the organ being studied or with that of any other organ; it requires the opening of no blood vessels, the injection of no foreign substances.

(3) It has a relatively rapid response time (much faster than that of Verzar, probably faster than those of Rein and of Kramer, but, at the present time, considerably slower than that of Anrep).

Against it:

(1) The present method is indirect, measuring blood flow by means of the speed with which it supplies oxygen to the muscle.

(2) The blood flow measurements are not continuous but intermittent, requiring either a series of contractions or a temporary clamping off of the arterial supply for a measurement.

(3) It can give only relative values, not absolute ones.

(4) Its data are obtained by a new and fairly complicated technique.

The present unsatisfactory position may be summarized as follows: experiments involving perfusion and opening of blood vessels (earlier workers, Anrep) have revealed a diminution or stoppage of blood flow during tetanic contraction, whereas those in which the circulation has been kept intact (direct: Rein; semi-direct: Kramer; indirect: Millikan) have shown a continued or even a slightly increased flow.

B—*Speed of Utilization of Oxygen during Contraction, Response Delay less than $\frac{1}{5}$ sec.*

If we accept the conclusion reached in the preceding section, namely that the principal role of muscle haemoglobin is to provide a store of oxygen which will be called upon during sudden changes in the oxygen demand, rather than to smooth out fluctuations in its supply, we are at once faced with two important questions of muscle metabolism. The first is "How soon after the contraction starts does the increased oxygen demand *begin* to make itself felt?", and the second: "How rapidly is oxygen used up during contraction?" The first question deals with the rate at which the chain of chemical processes ending with oxygen demand gets under way, i.e. its latent period, while the second is concerned with how long it takes these processes to play themselves out.

To the first question, we can at present only make the partial answer, "In less than $\frac{1}{5}$ sec.", since this is the time of response of our measuring instrument, the records showing the onset of oxygen consumption at the same time as the onset of contraction. Further refinements in the apparatus, which are at present being developed, may enable us to reduce this estimate, but should not require us to increase it, for any sluggishness of response in an instrument which is critically damped tends to round off any sudden changes, never to accentuate them.

At first sight these results would appear to be in conflict with those of Verzar (1912), who sometimes found a decrease in oxygen consumption during a long tetanus, and always observed a large increase after it. The time at which increased oxygen consumption begins is something which one could not hope to learn from his experiments, because of the time lag of the method, and to the change of blood flow which he often observed under the conditions of his experiments. Verzar states "no conclusions can be built upon this decreased oxygen use, as it is certainly caused by change of blood flow".

The present findings are in confirmation of Kramer's (1936*b*) measurements on the oxygen content of venous blood from a dog's limb, made with his continuously recording photocell method. These gave a time lag of 1·5–2 sec. between the time of contraction and the increased oxygen consumption, and this is about the length of time which should be required for the blood to travel from the muscle capillaries to the recording element on the vein.

Absolute Rates of Oxygen Consumption before and during Contraction.

With regard to the second question we are in a more fortunate position, for the instrument can give us quantitative evidence in regard to oxygen consumption during the first few seconds of contraction. It should be noted that though the "time of response", i.e. the shortest interval in which it is possible to say: "something is happening and in this direction but we don't yet know how much" is $\frac{1}{5}$ sec., the "time for quantitative registration" is about the same as the dead beat period of the galvanometer or 1·2 sec. Our rates of oxygen consumption, given in italics in Table II, are therefore calculated on the basis of the changes in muscle haemoglobin saturation during the first 2 or 3 sec. of contraction. They are obtained by multiplying the initial slopes of Plates 9, 10, and 11 by the oxygen content of the muscle haemoglobin in the tissue, which has been estimated as follows. A number of water extracts of the minced muscle gave an average value of about 5 mg. Mgb/g. fresh tissue, as determined spectrophotometrically, and this value agrees well with Whipple's (1926) corresponding figures for dogs, but it is probably only accurate to about 25 %. Since 5 mg. of the pigment can combine with $5 \times 22{,}400/16{,}700 = 6{\cdot}65$ mm.^{3} O_2, the rate of oxygen utilization in mm.^{3} O_2/g. tissue/sec. is simply the slope of the deoxygenation curve × 6·65. Example: resting consumption: Plate 9*a*: 0·98 % Mgb/sec., which equals 0·0098 × 6·65 = 0·064 mm.^{3} O_2/cm./sec.

Since no measurements were made in the present series of experiments on oxygen consumption by conventional methods, the best available check on our results is a comparison of the present data with previously obtained values on similar tissues. Such figures have been collected in Table II. Verzar's values for oxygen consumption during a single contraction, which are about ten to twelve times lower than our figures, have not been included in the table, because they are not really comparable. Due to the time delay inherent in Verzar's method of measurement, some of the oxygen measured during contraction does not correspond to metabolic changes taking place at that time. Verzar's resting values do, however, correspond to ours and it is seen that the figures obtained for cat gastrocnemius are in very good agreement with our soleus data.

TABLE II—OXYGEN CONSUMPTION OF RED MAMMALIAN MUSCLE MEASURED INSTANTANEOUSLY BY "METABOLIC MICROSCOPE" AND COMPARED WITH RESULTS BY OTHER METHODS

All figures in mm.^{3} O_2/g. muscle/sec.	Average value	Maximum value
(1) *Resting Metabolism*		
Cat soleus; present experiments	*0·064*	*0·105*
Cat gastrocnemius; blood collection (Verzar 1912)	0·075	0·142
(2) *Activity*		
Cat soleus; present experiments	*1·32*	*3·5*
Dog heart (Evans and Matsuoka 1915)	0·90	2·7
Dog heart; normal activity (Starling 1930)	1·25	—
Man running; "steady state of severe exercise" (A. V. Hill 1928)	—	3·3
Dog gastrocnemius; rhythmic stimulation (Barcroft and Kato 1915)	1·00	2·16

What is more significant, we find that our instantaneously measured rates for activity agree well with the previously measured values for the *overall* consumption of active red muscles operating in a normal repetitive way at high efficiency, whether the muscles in question be those of dog's heart, man's legs or dog's gastrocnemius. This agreement lends support to the theory that the easily mobilizable and quickly utilized oxygen, which we are here measuring, constitutes the great bulk of oxygen required by repetitively contracting—but not overworked—mammalian muscle.

C—*Normal and Emergency Mechanisms of Muscular Contraction*

It appears from the present work that the normally contracting mammalian muscle operates aerobically, in the sense that if oxygen is available it makes use of it within a fraction of a second after the performance of work. Furthermore, the special provision for oxygen storage in the form of muscle haemoglobin suggests that this quick, aerobic, debtless mechanism is the efficient and satisfactory one. On the other hand, it has long been clear that a muscle *can* operate for some time in the absence of oxygen, and it is reasonable to assume that for this emergency service an emergency mechanism is brought into play, presumably of lower efficiency than the normal one, but enabling the muscle to develop an oxygen debt. In other words, the muscle can run off its accumulators if it has to, charging them up afterwards, but it prefers to operate by direct drive if it can. If this general picture is correct—and it is admittedly a speculative one—we are tempted to try and relate these two mechanisms with the known thermal and chemical changes which accompany contraction.

The delayed oxidative recovery heat measured by Weizsäcker (1914), by Hill and Hartree (1920), and again by A. V. Hill (1928), when a muscle contracts in the presence of ample oxygen, appears at first sight difficult to reconcile with the very rapid oxygen consumption found here, but as Hill has pointed out (personal communication), the time relations for mammalian muscle at 37° C. are very much shorter than those for amphibian muscle at room temperature; it is quite likely that events are so telescoped in the normal cat's soleus that recovery heat appears within a fraction of a second of the contraction. Cattell and Shorr (1932) have in fact shown that even at the same temperature, dog's scalenus muscle has a more rapid oxidative recovery heat than frog's sartorius.

By determining the time course of oxygen consumption in a muscle, we are provided with data which we can use to apply the kinetic test to any suggested mechanism of contraction. Whatever may be the chain of chemical processes connecting muscular contraction with the uptake of oxygen, and however many intermediate steps it may contain, the present experiments show that in normal contraction the whole chain gets into action in a fraction of a second. If we can prove that any proposed reaction can only take place more slowly than this, we may exclude it from playing an important part in the main chain.

Lactic acid may be thus excluded on the basis of the present findings, unless we assume that lactic acid can be removed oxidatively from the muscles several hundred times more rapidly than from the body as a whole, and that it does not diffuse out quickly from the muscles into the blood stream. There is, however, good evidence that neither of these assumptions is correct (Margaria, Edwards and Dill 1933; Sacks and Sacks 1935*b*). The conclusion that lactic acid formation, at least on any considerable scale, is to be regarded as a part of the emergency mechanism, and not directly associated with normal activity, is not in contradiction to modern ideas of muscle biochemistry.*

The resynthesis of phosphocreatine appears also to proceed at a much more leisurely pace in the muscle than the oxygen consumption (Sacks and Sacks 1935*a*), suggesting that this process too is not directly an oxidative one, but the evidence here is more scanty and less directly applicable to the cat's soleus. On the basis of present findings alone we should be rash to eliminate the phosphocreatine reaction as a part of the normal mechanism. About the rates of resynthesis of adenylic triphosphate, we still know too little to attempt to subject it to the kinetic test.

For the rigorous application of the kinetic test, two conditions are essential. First, we must know how rapidly the reactions can take place in the animal being studied under the same conditions as in the muscle experiments, and secondly we must know just how rapidly oxygen is being consumed at the moment of contraction, not just its limiting rate. The first requires parallel chemical experiments on the muscle which is being studied spectroscopically, the second requires pushing up the time resolving power of the "metabolic microscope". The research is now being pursued in these two directions, and more extensive speculation on the present platform of observed data is perhaps premature.

This paper owes much to discussions with a host of kind people who have been good enough to go over portions of it with me and give generously of their counsel and advice. Specific suggestions, of which I have made free and grateful use, have been made by Professor Sir Joseph Barcroft,

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* A considerable part of the "alactacid debt" of Margaria, Edwards and Dill (1933) is undoubtedly not a true metabolic debt but a replenishment of the body's oxygen stores, which have been depleted by the instantaneous call for oxygen, described in this paper. A rough calculation suggests, however, that the total amount of the known oxygen stores of the body (oxygen in solution, muscle haemoglobin, blood haemoglobin, and lungs) can account at the most for only about a half of the alactacid debt of nearly 3 l. found by these authors. The calculation follows: 70 kg. man; solution oxygen, less than 0·2 l.; Mgb, less than 0·4 l.; Hb, less than 0·8 l.; lungs, less than 0·4 l.; total, less than 1·8 l.; and all the estimates have been generous. We conclude that it would not be at all surprising to find an anaerobic mechanism, in addition to the lactic acid one, which played a part in the "emergency" mechanism of the present classification.

Professor A. V. Hill, Dr. K. Kramer, Professor A. von Muralt, Dr. D. M. Needham, Dr. F. J. W. Roughton and Professor F. Verzar. A part of the experimental work was made possible by a grant from the Rockefeller Foundation.

SUMMARY

Method.

A photoelectric arrangement is described by means of which the degree of oxygen saturation of the intracellular muscle haemoglobin of a cat's soleus muscle may be measured instantaneously and recorded continuously.

It provides both a *chemically-specific* and *time-sensitive* method of measuring metabolism of red muscle. Measurements can be made without interfering with either the nerve supply or the blood supply to the tissue.

The response lag of the instrument is about 0·2 sec.; the measurement time about 1·3 sec. Controls have shown that the presence of blood haemoglobin in the muscle capillaries causes an error of less than 10% in the readings, and that opacity changes in the muscle during contraction produce errors of about the same magnitude.

Experimental Results.

Muscle haemoglobin acts as a short time oxygen store to tide the muscle over from one contraction to the next.

When contraction takes place, the oxygen demand starts to rise in less than 0·2 sec., reaches its maximum value inside of 1 sec. from the onset of contraction, and falls nearly to its resting value within 10 sec. of the end of contraction, when the contraction has only been of a few seconds duration.

The initial rate of oxygen consumption of cat's soleus muscle during maximal tetanic contraction, as measured with the "metabolic microscope" is from 1·3 to 3·5 mm.^{3} of O_2 per gm. of muscle per sec. These figures are in agreement with previous measurements made by classical methods.

Blood flow through the soleus muscle appears not to be greatly affected by maximal tetanic contraction, whether the muscle be stimulated directly or by its nerve; the flow is certainly not completely stopped.

These results are in agreement with those of Rein and Kramer; they differ from those of Anrep.

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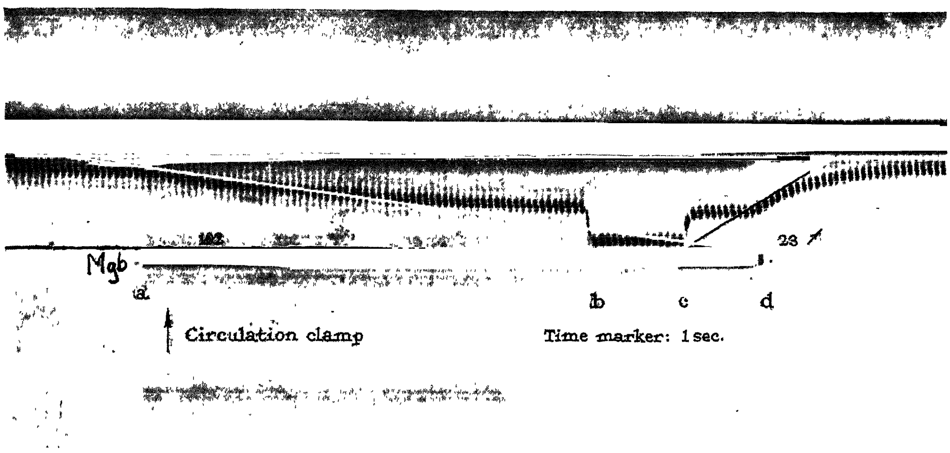
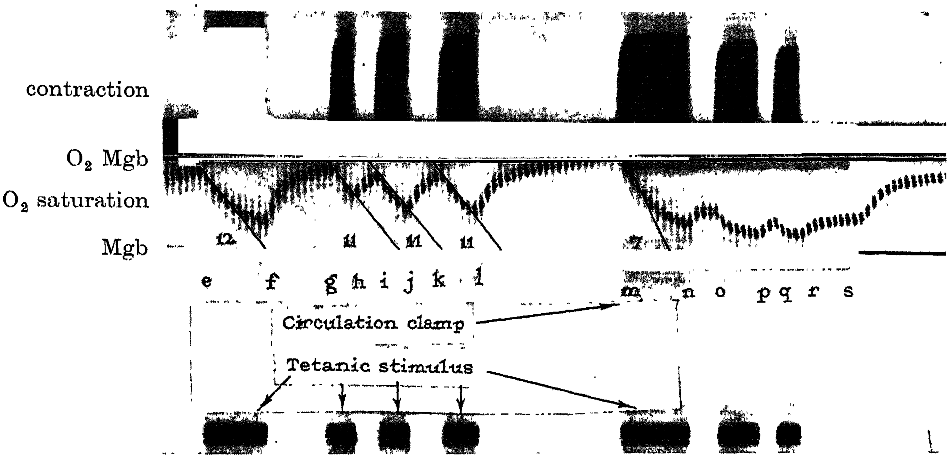
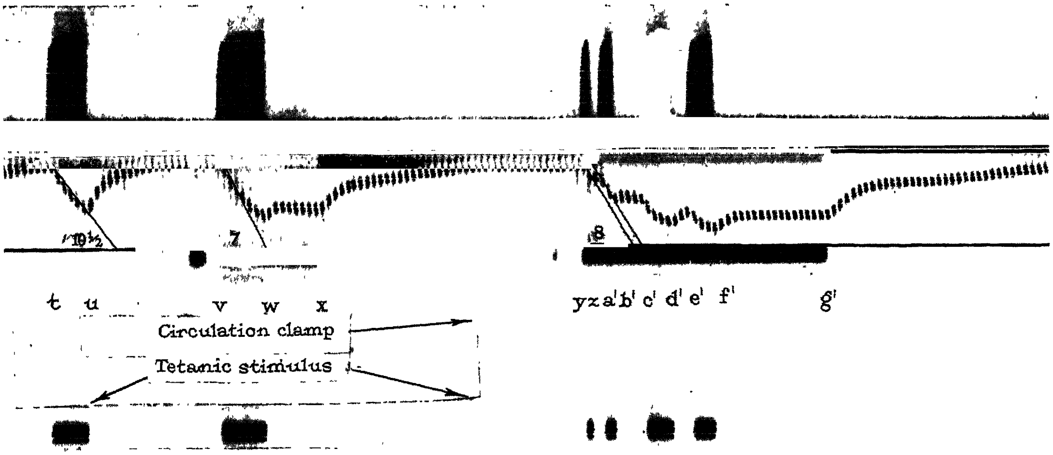
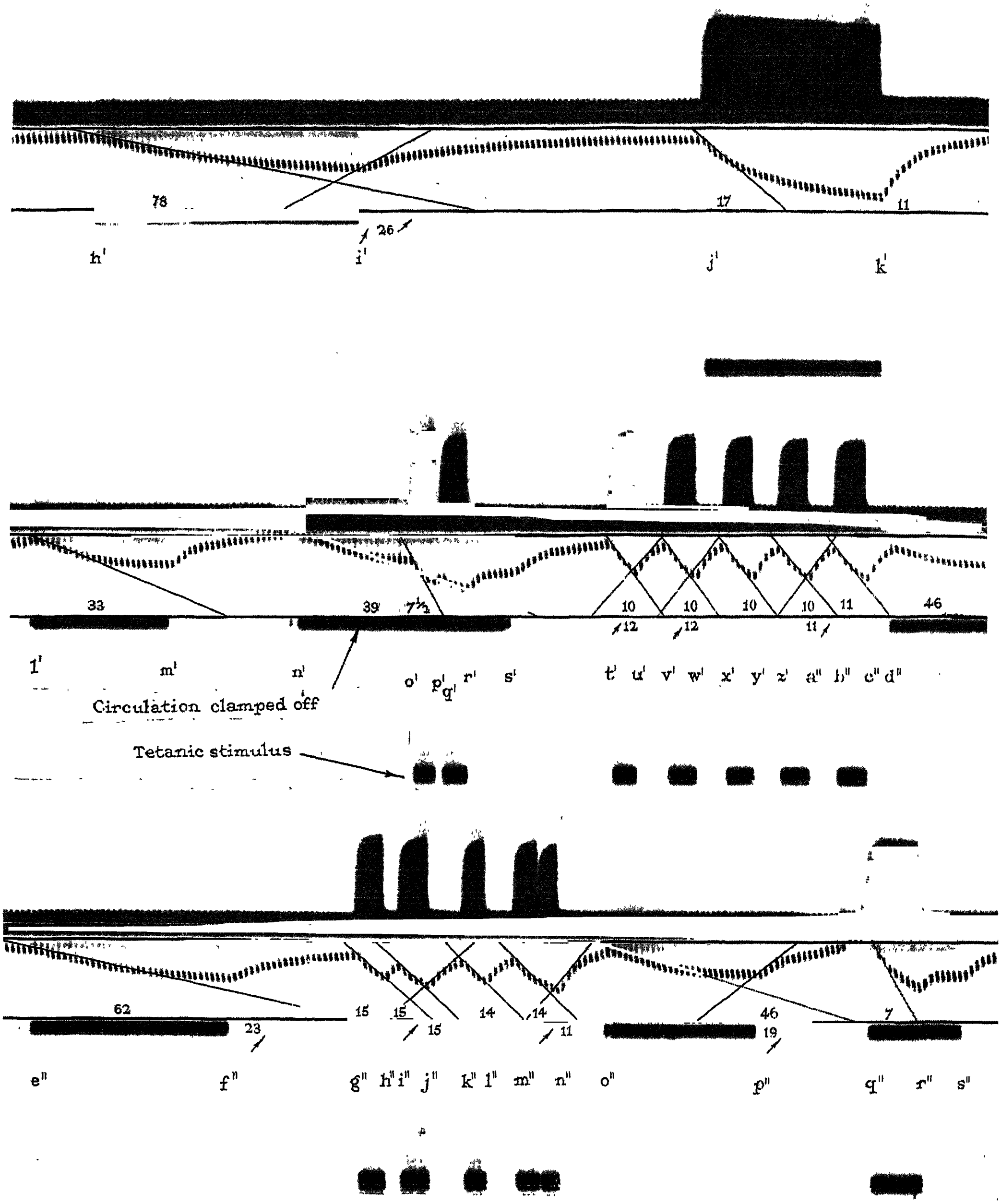

102
23
Mgb
a
b
c
d
Circulation clamp
Time marker: 1 sec.


contraction
O_2 Mgb
O_2 saturation
Mgb
12
11
11
11
7
e f g h i j k l
m n o p q r s
Circulation clamp
Tetanic stimulus


10½
7
8
t u v w x
y z a' b' c' d' e' f' g'
Circulation clamp
Tetanic stimulus


78
26
17
11
h'
i'
j'
k'
33
39
7½
10
12
10
12
10
10
11
11
11
46
l'
m'
n'
o'
p'
q'
r'
s'
t'
u'
v'
w'
x'
y'
z'
a''
b''
c''
d''
Circulation clamped off
Tetanic stimulus
62
23
15
15
15
14
14
11
46
19
7
e''
f''
g''
h''
i''
j''
k''
l''
m''
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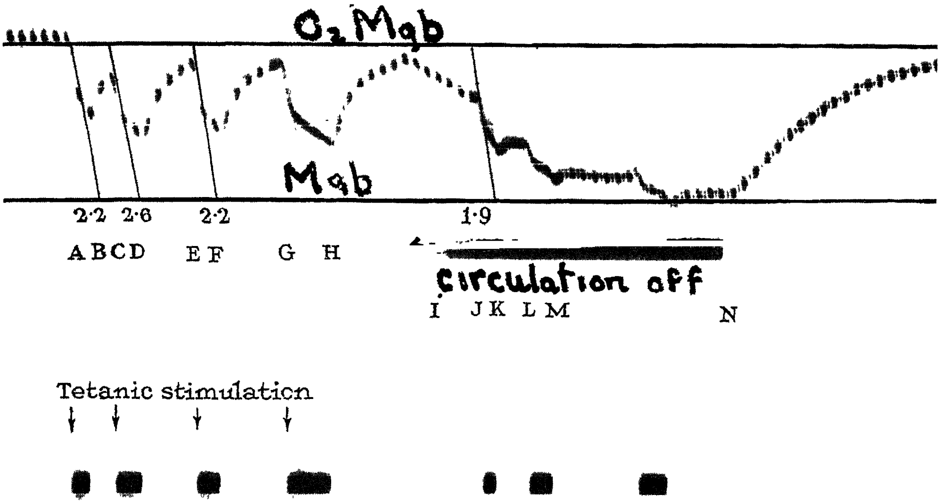
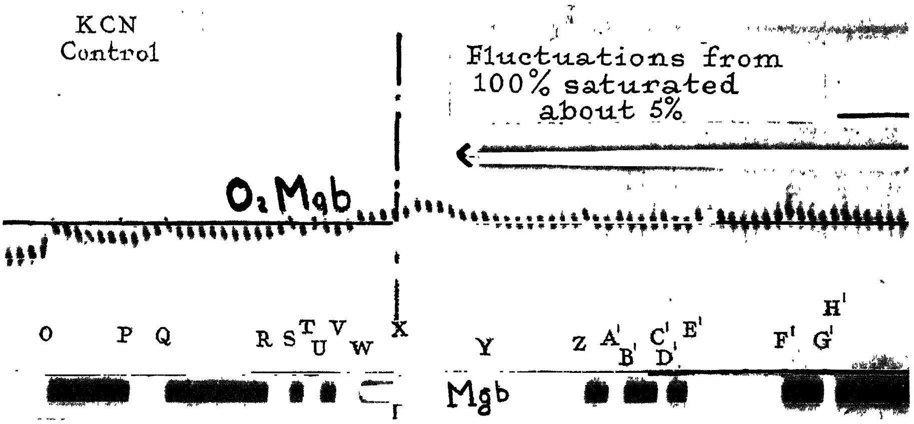
s''


O₂Mgb
Mgb
2·2 2·6 2·2 1·9
A B C D E F G H
circulation off
I J K L M N
Tetanic stimulation


KCN
Control
Fluctuations from
100% saturated
about 5%
O₂Mgb
O P Q R S T U V W X
Y
Z A' B' C' D' E' F' G' H'
Mgb

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DESCRIPTION OF PLATES

Plates 9–11

Records of muscle metabolism during rest, activity, and recovery, using muscle haemoglobin as intracellular oxygen indicator. Nervous stimulation, 50 Hertz.

Plates 9, 10. A single experiment. The record is continuous except for a 2 min. rest between *d''* and *e''*.

Plate 11. Cyanide control. (*a*) Portion of record immediately before, and (*b*) immediately after the injection of 0·5 ml. of 1 % KCN into the muscle.

581.12: 581.134.4

Respiration of Barley Plants

III—Protein Catabolism in Starving Leaves

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In an earlier paper (Yemm 1935) the results of analytical and gasometric experiments with starving barley leaves were described. These experiments indicated that the respiratory mechanism was considerably modified under conditions of extreme starvation: the rapid breakdown of carbohydrates, conspicuous at first, may play only a minor part in the later stages. It was suggested that protein catabolism is important in the respiration of highly starved and yellow leaves. The evidence for this conclusion was drawn primarily from observations of the respiratory quotient; such data are notoriously difficult to interpret and alone they do not lead to any satisfactory certainty. In the present case further information could be gained by biochemical examination of the transformation of proteins and other nitrogenous compounds occurring during progressive starvation. By this means the preliminary analysis of the living system by gasometric methods could be extended.

It is known that loss of protein occurs in plants under conditions of carbohydrate shortage. Onslow (1931) has collected together analytical data which show this in both etiolated seedlings and isolated organs, while the researches of Chibnall (1922*a*, 1924*b*), Mothes (1926), and Vickery, Pucher, Wakeman and Leavenworth (1933) have provided information especially for detached leaves. However, any detailed comparison of the results available with the starving barley leaves is obviously hazardous, since differences, both of the leaf type and of starvation conditions, may lead to confusion. Therefore, biochemical data have been sought for barley leaves starved under the controlled conditions previously used: a description of the analytical methods and results is given in this paper.

I—ANALYTICAL METHODS

The instability of many of the products of protein breakdown made immediate analysis of the starving leaves desirable: drying the leaves or storage in alcohol may involve considerable change in the nitrogenous fractions, as shown by Chibnall (1922*b*) and Thomas (1927). This consideration caused the adoption of methods which could be carried out rapidly, as much information could be gained only by taking samples at fairly frequent intervals. The procedure finally adopted aimed at the determination of the following fractions:

(1) Total insoluble nitrogen.
(2) Total water-soluble nitrogen.
(3) Free ammonia nitrogen.
(4) Stable amide nitrogen.
(5) Unstable amide nitrogen.
(6). Total amino nitrogen.

Estimation of (1) *Total Insoluble Nitrogen and* (2) *Total Water-Soluble Nitrogen*

The separation of the nitrogenous constituents of the leaves into water-soluble and insoluble fractions was effected by the method of Chibnall (1924*b*), involving extraction with ether water. Three successive extractions with slightly acid ether water (0·002 N HCl) were used, the leaf material being pressed (2 tons/sq. in.) between tinned plates on a small hydraulic press after each extraction. The complete water extract was boiled rapidly for 2–3 min. to coagulate the water-soluble protein, and then filtered. The coagulated protein removed by filtration together with the leaf residue was used for the estimation of total insoluble nitrogen by standard incineration with sulphuric acid and determination of ammonia by the micro-Kjeldahl method. After making up to a convenient volume, aliquots of the clear yellow brown extract were used for estimation of total insoluble nitrogen and the other soluble fractions (3–4). Total soluble nitrogen was determined by the standard incineration and micro-Kjeldahl method as above.

Various points in the extraction method have been tested. On numerous occasions the first three were followed by three further extractions. Analysis showed that 94–7 % of the soluble nitrogen was in the first three extracts.

TABLE I—EXTRACTION OF SOLUBLE NITROGEN WITH ETHER WATER

Extract no.	Hours starvation	First three extractions (mg. N)	Second three extractions (mg. N)	% N in first three extractions
1	12	2·72	0·16	94
2	60	4·45	0·10	97
3	84	5·20	0·23	95

By a full analysis of the different solutions it was found that the quantities of ammonia, amide and amino nitrogen present in the second three extractions were so small as to be hardly beyond the limits of experimental error.

Thomas (1927) has found that with some types of leaf more soluble nitrogen is extracted by the boiling water method than with Chibnall's. These two methods have therefore been compared in the analysis of similar samples of barley leaves. For the boiling water extraction the following procedure was used. The leaves were cut into small strips and on to them were poured 50 c.c. of boiling water. After standing in a boiling water bath for a few minutes to ensure complete killing the extraction was allowed to proceed for 30 min. in the cold. The leaves were then pressed on the hydraulic press and re-extracted three times as before. Three experiments showed that in every case slightly more soluble nitrogen was obtained by the ether water method. The results of one experiment are shown in Table II.

TABLE II—COMPARISON OF EXTRACTION METHODS

	Ether water Mg. N/1 g. fresh weight	Boiling water Mg. N/1 g. fresh weight
Total insoluble nitrogen	2·72	3·06
Total soluble nitrogen	2·85	2·65
Free ammonia nitrogen	0·03	0·03
Amide nitrogen	0·19	0·19
Total amino nitrogen	1·37	1·32

From the results it is apparent that the difference in total soluble nitrogen is not due to variation in the amount of ammonia amide on amino nitrogen extracted: very little difference could be detected in these fractions whichever method was used. However, the modifying effect of the extraction treatment on the solubility of some of the other nitrogenous constituents emphasized the importance of carefully standardizing the extraction method in order that comparable estimates of total soluble nitrogen should be obtained. In all experiments to be described here the ether water method, as already described, has been used.

The use of various precipitants for the removal of soluble protein nitrogen from the crude ether water extract has been compared with the method of coagulation by boiling. Trichloracetic acid and colloidal iron were used under conditions recommended by Thomas (1927). Aliquots of the same crude extract were treated by the different methods and the total nitrogen content and other nitrogenous fractions then estimated. Each of two experiments carried out in this way showed that the various methods of protein removal had very little effect on either the total soluble nitrogen determination or the other soluble fractions.

TABLE III—COMPARISON OF PROTEIN PRECIPITATION METHODS

	Total mg. nitrogen/1 g. fresh weight		
	Boiling	Colloidal iron	Trichloracetic acid
Total soluble nitrogen	3·05	3·13	3·10
Ammonia nitrogen	0·05	0·04	0·05
Stable amide nitrogen	0·18	0·17	0·17
Unstable amide nitrogen	0·25	0·27	0·26
Total amino nitrogen	1·58	1·58	1·60

(3) *Estimation of Free Ammonia*

The micro-Kjeldahl distillation apparatus as modified by Parnas and Heller (1924) was used. The distillation was carried out with magnesium oxide at 40° C. for 15 min. under conditions similar to those adopted by Chibnall and Westall (1932).

Trial experiments with weighed quantities of ammonium sulphate gave yields of 98–100 % of the total amount of ammonia added.

(4) *Estimation of Total Amide Nitrogen*

The most important amides likely to be present in plant extracts are asparagine and glutamine: the methods of separate determination depending on their different susceptibilities to hydrolysis and suggested by Chibnall and Westall (1932) have been used. After hydrolysis of these amides by heating with dilute acid or acetate buffer the solution was transferred to the micro-Kjeldahl apparatus for estimation of the free ammonia,

TABLE IV—HYDROLYSIS OF PURE ASPARAGINE

Extract no.	Hours hydrolysis	Acid normality	Mg. amide nitrogen (found)	Mg. amide nitrogen (added)	% hydrolysis
Control	—	—	0·02	1·76	—
1	2	N	1·42	1·76	81
2	3	N	1·61	1·76	91
3	4	N	1·66	1·76	94
4	2	2 N	1·64	1·76	93
5	3	2 N	1·69	1·76	96
6	4	2 N	1·72	1·76	98
7	5	2 N	1·71	1·76	97
8	3	Acetate *p*H 4	0·08	1·76	5

as already described. The acid hydrolysis was carried out under conditions slightly different from those used by Vickery and Pucher (1931)—the acid solution with a reflux condensed was heated in a boiling water bath. Trial of various acid concentrations and heating periods was made with a pure B.D.H. preparation of asparagine, since this is the more stable of the two amides.

The analyses showed that after heating for 4 hr. with 2 N sulphuric acid no further production of free ammonia could be detected, while heating with an acetate buffer *p*H 4, the conditions used in glutamine estimation, caused relatively little hydrolysis. A similar trial was made with an extract from barley leaves—aliquots being subjected to varying periods of hydrolysis in presence of N and 2 N sulphuric acid.

TABLE V—ACID HYDROLYSIS OF LEAF EXTRACT

Extract no.	Hours hydrolysis	Acid normality	Mg. amide nitrogen	% hydrolysis
1	2	N	0·68	84
2	3	N	0·70	87
3	4	N	0·75	94
4	5	N	0·74	93
5	1	2 N	0·63	78
6	2	2 N	0·68	84
7	4	2 N	0·80	99
8	5	2 N	0·81	100

As found with pure asparagine very little further increase in ammonia resulted from hydrolysis more severe than 4 hr. with 2 N sulphuric acid. On the basis of these experimental results total amide nitrogen in the extracts has been estimated by hydrolysis for 4 hr. with 2 N acid and subsequent measurement of the increase in ammonia, and compared with the initial value in the untreated extract.

(5) *Estimation of Unstable Amide Nitrogen*

The method described by Chibnall and Westall (1932), involving hydrolysis with an acetate buffer *p*H 4·0 for 3 hr. in a boiling water bath, has been extensively used. By this treatment 46 % of the nitrogen of the more unstable glutamine is split off as ammonia, while the more stable asparagine is relatively little affected. Trials were also made with phosphate buffers (*p*H 8) but these were unsatisfactory, since full recovery from pure ammonia solutions could not be obtained, probably owing to the formation of the insoluble magnesium ammonium phosphate during distillation (Vickery, Pucher, Clark, Chibnall and Westall 1935).

Over a hundred analyses of barley leaf extracts have shown in almost every case a considerable increase in free ammonia on hydrolysis at *p*H 4. The special reactions of glutamine, described by Chibnall and Westall (1932), made it possible to decide with greater certainty whether this compound was present as distinct from other nitrogenous constituents which might yield ammonia under similar conditions. These authors showed that when glutamine was hydrolysed at *p*H 4 there occurred an increase of ammonia and loss of amino nitrogen, as estimated by the Van Slyke method, such that:

$$\frac{\text{Loss of amino nitrogen}}{\text{Increase of ammonia nitrogen}} = 1{\cdot}9.$$

Therefore, the change of the amino and amide fractions on hydrolysis have been estimated in extracts made from leaves at different stages of starvation.

TABLE VI—CHANGES OF AMINO AND AMMONIA NITROGEN ON HYDROLYSIS AT *p*H 4

		Mg. nitrogen				
		Initial values		After hydrolysis		
Extract no.	Hours starvation	Ammonia	Amino	Ammonia	Amino	Loss of amino nitrogen/ Increase in ammonia nitrogen
1	6	0·08	2·32	0·18	2·04	2·8
2	12	0·05	3·23	0·31	2·72	2·0
3	48	0·08	6·32	0·99	4·76	1·7
4	60	0·15	5·60	0·83	4·20	2·0
5	72	0·27	8·00	1·61	5·67	1·7
6	108	0·38	5·60	0·94	4·40	2·1
7	120	0·70	2·91	1·26	1·87	1·9
8	130	3·31	5·20	3·80	4·12	2·2
9	144	3·94	7·60	5·51	4·68	1·8
10	168	3·01	3·79	3·63	2·14	2·0

With extracts made from leaves soon after their detachment from the plant (Table VI, No. 1) the changes of both the ammonia and amino nitrogen were so small that the experimental errors in determining the ratio were relatively large, but in all other extracts when large differences occurred a close similarity was found between the changes observed and those to be expected if glutamine were present. The mean value of the ratio from the above estimations with leaves starved for periods from 6 to 168 hr. was very close to 1·9, characteristic of glutamine.

Another property of glutamine which renders its identification possible is the loss of amino nitrogen on hydrolysing with sulphuric acid; Chibnall and Westall (1932) have shown that if this occurs the ratio loss of amino nitrogen on acid hydrolysis/total glutamine amide nitrogen should be close to unity.

The estimations necessary to determine this ratio were made on a series of different extracts from starving leaves, and the results are summarized in Table VII.

TABLE VII—CHANGE IN AMINO NITROGEN ON ACID HYDROLYSIS

		Mg. nitrogen			
		Amino nitrogen		Total Glutamine amide nitrogen (pH 4 hydrolysis)	Loss of amino nitrogen/ Glutamine amide nitrogen
Extract no.	Hours starvation	Before hydrolysis	After hydrolysis		
1	60	5·19	4·27	0·92	1·0
2	90	5·73	4·60	1·28	0·9
3	90	6·41	4·87	1·28	1·2
4	120	4·49	3·64	0·91	0·9
5	140	3·76	3·34	0·41	1·0

The possibility that the hydrolysis of peptides present in the extracts might interfere seriously with the determination of the loss of amino nitrogen on acid hydrolysis has been considered. In the analysis of one extract made from starving leaves hydrolyses of different severities were compared: change of amino nitrogen was estimated after varying periods of hydrolysis with both N and 2 N acid to see if any appreciable differences occurred. The extract No. 4 from Table VII was used for the tests.

TABLE VIII—EFFECT OF VARIATION IN ACID HYDROLYSIS ON THE DETERMINATION OF AMINO NITROGEN

			Mg. nitrogen			
			Amino nitrogen		Total glutamine amide nitrogen (pH 4 hydrolysis)	Loss of amino nitrogen/ Glutamine amide nitrogen
Extract no.	Hours hydrolysis	Acid normality	Before hydrolysis	After hydrolysis		
1	3	N	4·49	3·72	0·91	0·8
2	4	N	4·49	3·76	0·91	0·8
3	5	N	4·49	3·72	0·91	0·9
4	2	2 N	4·49	3·50	0·91	1·1
5	3	2 N	4·49	3·70	0·91	0·9
6	4	2 N	4·49	3·64	0·91	0·9

No progressive change in the estimate of amino nitrogen could be detected and the value of the amino/amide ratio (column 7) was not seriously affected.

It is clear from the data summarized in Tables VI–VIII that the reactions of leaf extracts with respect to both *p*H 4 and acid hydrolysis were very similar to those expected if glutamine were present. However, recently Melville (1935) has shown that these reactions are also shown to some extent by some labile glutamine peptides. The possibility of such compounds being present is not excluded by the extraction methods, and therefore the general term unstable amide has been used in description of the fraction estimated by hydrolysis at *p*H 4. To obtain more precise information direct methods of chemical isolation will be necessary.

Since this work was finished, Vickery and others (1935) have recommended hydrolysis at *p*H 6·5 for the estimation of glutamine in plant extracts. A more complete hydrolysis, and less likelihood of interference by other possible nitrogenous constituents, are the chief advantages of such a method. With the procedure used here it seems certain that any serious error caused by other unstable nitrogenous compounds would have been exposed by the different quantitative tests which were made. It is very unlikely that a combination of other substances such as ureides and hydroxyglutamic acid could give the consistent amino/amide ratios which were observed with samples during progressive starvation; as will be shown later, rapid fluctuations of both glutamine and other nitrogenous constituents occurred, but the reactions of the fraction estimated at *p*H 4 were always similar to those of glutamine or glutamine peptides.

Despite its instability it is improbable that any serious hydrolysis of unstable amide occurred during preparation of leaf extracts for analysis. These extracts during progressive starvation had a *p*H 6–6·5 and were boiled for 2–3 min. to coagulate the soluble proteins. However, in many experiments although relatively large quantities of unstable amide were present the ammonia content of the extracts was consistently low.

(6) *Estimation of Amino Nitrogen*

On the basis of the results of Richardson (1934) the micro-Van Slyke method (Van Slyke 1914) was used for the determination of amino nitrogen in the coloured extracts from barley leaves. The estimation was always made after the removal of ammonia by distillation, the residue in the micro-Kjeldahl distillation flask being acidified by the addition of sufficient glacial acetic acid to dissolve the excess magnesium oxide. After dilution to a convenient volume 2 c.c. aliquots were used in the reaction chamber, shaking for 10 min. at 17–21° C. The mean of a large number of blank determinations had a value of 0·15 ± 0·02 c.c. while the agreement between duplicates was usually within 0·04 c.c. gas.

Rahn (1932) and Hume (1935) have shown that the presence of tannins interferes seriously with the estimation of amino nitrogen. Qualitative tests indicated that tannins were present in barley leaf extracts, although the darkening with ferric chloride was only slight. The method of removal recommended by Hume (1935) was applied in a series of experiments to test whether the amount of tannin was sufficient to cause appreciable error. This precipitation method involved standing the extract with potassium dichromate at *p*H 3·8–4·4, and since unstable amides were present their breakdown might affect the amino nitrogen value. Therefore, a control was necessary in which an aliquot of the extract was treated in a similar manner, except for the omission of the precipitant. The results of the analysis of extracts made from leaves at different stages of starvation are shown in Table IX.

TABLE IX—EFFECT OF TANNIN REMOVAL ON THE DETERMINATION OF AMINO NITROGEN BY THE VAN SLYKE METHOD

Extract no.	Hours starvation	c.c. Nitrogen	
		Dichromate	Control
1	0	0·54	0·52
2	24	1·26	1·23
3	48	2·21	2·20
4	72	2·45	2·42
5	96	2·71	2·72

The differences caused by the precipitation of tannins was always within the limits of the experimental error, and it is unlikely that these substances were present in sufficient quantity to interfere seriously with amino-nitrogen determination. Additions of pure B.D.H. tannin were made in some cases and it was always found that the interference in the Van Slyke determination could be quantitatively removed by dichromate precipitation under the conditions used.

Stuart (1935) found that a considerable reduction in the estimate of apparent amino nitrogen occurred in some plant extracts on treatment with purifying agents. A maximum effect, varying from 6 to 85 % of the total amino nitrogen, was always observed when calcium oxide was used as the absorbent. The reagent was used for the micro-Kjeldahl distillation and then removed by filtration, the solution being acidified with acetic acid as before. This treatment was compared with the standard magnesium oxide method for removal of ammonia and amino nitrogen estimation (Table X).

Table X—Comparison of Methods of Ammonia Removal and Amino Nitrogen Estimation

Extract no.	Hours starvation	Mg. O treatment		Ca(OH) treatment	
		Ammonia nitrogen mg.	Van Slyke c.c. gas	Ammonia nitrogen mg.	Van Slyke c.c. gas
1	12	0·55	0·51	0·60	0·51
2	36	0·70	0·72	0·70	0·74
3	60	1·20	1·01	1·20	0·99
4	84	2·80	1·22	2·80	1·18

No significant difference in the ammonia estimations could be detected, indicating that the breakdown of glutamine with the stronger alkali was not important, while only small and variable differences were found in the production of gas in the Van Slyke apparatus. It seems probable therefore that errors owing to the presence of tannins or other impurities were relatively small.

As summarized by Richardson (1934), the Van Slyke method suffers from serious defects in the non-quantitative reaction with certain amino acids and peptides. A correction for some of these irregularities can be made when separate determination is available, and the Van Slyke determination has been used for estimation of amino nitrogen after the deduction of the appropriate glutamine and asparagine fractions. Although no very exact quantitative significance can be attached to the amino nitrogen values, the method seems to be the most reliable at present available for comparative purposes.

II—Addition of Nitrogenous Compounds to a Leaf Extract

The analytical methods were further tested by the addition of known quantities of different nitrogenous compounds to a leaf extract, and estimation of the amount recovered by analysis. An extract was divided into equal aliquots and to one of these were added known quantities of ammonium sulphate, asparagine and glycine: no pure preparation of glutamine was available. The results of the analyses and the amounts recovered are shown in Table XI.

In each fraction a fairly accurate recovery of added nitrogen was observed, the greatest difference being about 4 % in the case of total soluble nitrogen.

TABLE XI—RECOVERY OF NITROGENOUS COMPOUNDS

	Mg. nitrogen				
	Extract only	Extract + nitrogen	Difference found	Nitrogen added	Recovery %
(1) Ammonia	0·17	3·91	3·74	3·74	100
(2) Total amide	2·50	12·41	9·91	9·68	102
(3) Stable amide	0·33	10·24	9·91	9·68	102
(4) Unstable amide	2·17	2·17	0	0	—
(5) Amino	8·0	23·2	15·2	15·5	98
(6) Total soluble nitrogen	14·2	38·9	24·7	23·8	104

III—EXPERIMENTAL DETAILS AND RESULTS

The analytical methods already described were used in experiments during the two seasons 1934 and 1935: in all six separate experiments were made with starving barley leaves. A number of consistent features have been detected in the changes of nitrogenous constituents which occurred during starvation. Some differences between the separate experiments were apparent, but here the general characteristics present in all the experimental records will be especially emphasized. An important source of variation seems to depend on the age of the leaf at the time of its detachment from the growing plant; it is hoped that this factor will be treated more fully in a later paper.

On account of the important interrelation of carbohydrate and protein catabolism, indicated by the other analytical and gasometric experiments (Yemm 1935), precautions have been taken to reconstruct the conditions as faithfully as possible, so that a comparison of results could be made. The method of sampling and starvation conditions capable of control in the laboratory were similar to those already described; in all the experiments a continuous record of the rate of carbon dioxide production was kept, since this provides some check on the extent to which the changes of carbohydrates already recorded could be considered typical of these experiments. In some of the experiments during the 1935 season, estimates of the total soluble carbohydrates present in the leaves were made by slightly modified methods.

It was not possible to use plants of the Plumage Archer variety grown on the same experimental plot for all of these experiments; leaves were taken from the ordinary field crops grown in the vicinity of Oxford. During the two seasons leaves were taken from five different localities, plants of the Spratt Archer and Plumage Archer varieties being used for different experiments. The similarity of the results, both with respect to carbon dioxide production and nitrogen analyses, indicated that neither varietal nor cultural differences were serious enough to make a direct comparison difficult.

In two of the experiments (X and XI) a slightly different method of removal of samples was used. Three samples were enclosed in small light-proof chambers, as in previous experiments, and measurements of carbon dioxide production made by the Pettenkofer method; the other leaf strips were sealed into a larger darkened chamber, which could be immersed in the constant temperature water bath. No measurements of carbon dioxide production were made with the larger chamber but a fairly rapid stream of moist air was drawn through as in the other samples, and when a sample was required for analysis the chamber was temporarily disconnected. Comparative tests with the two methods showed that yellowing and browning of the leaves followed an essentially similar course, while no appreciable difference could be detected between the important nitrogenous constituents of leaves starved in the different ways. The individual features of the separate experiments (VI–XI) are summarized below:

Exp. VI—Plants of the Spratt Archer variety grown under field conditions on a good Cornbrash soil were used. The leaves were isolated on 2 July 1934, when the developing ears were just visible: about 700 leaf strips (upper half with the tip removed) were divided into twelve random samples of 50–60 leaves, for measurement of carbon dioxide production and analysis during progressive starvation. Samples were analysed initially and after 6, 12, 18, 30, 42, 54, 66, 90, 114, 138, 170 hr.

Exp. VII—The leaves were isolated on 17 July 1934, from plants of the same crop as that used for Exp. VI. The ears were developing rapidly and the lower leaves were all yellowing. About 400 leaf strips were divided into seven samples, one being removed for analysis initially and after 18, 36, 60, 108, 136, 184 hr.

Exp. VIII—Plants of the Plumage Archer variety raised from winter-sown seeds were used: 500 leaves were detached on 27 May 1935 when the plants were 1½–2 ft. high, and the ears just becoming visible. Random samples of about 60 leaves were used for the measurements of carbon dioxide production and for analyses, which were made initially and after 24, 42, 66, 90, 120, 144, 178 hr.

Exp. IX—About 450 leaves were detached from Plumage Archer plants raised from spring-sown seeds on 16 June 1935. Seven random samples were analysed initially and after 24, 48, 72, 96, 120, 168 hr.

Exp. X—Field-grown plants (Spratt Archer) were used, the leaves being detached on 16 July 1935, when the ears were visible and leaves lower than the third were beginning to yellow. The upper half with the tip removed was isolated and the strips divided into seven random samples. Analyses of the starving leaves were made initially, and after 20, 48, 64, 88, 108, 132 hr.

Exp. XI—The leaves were isolated from Spratt Archer plants on 27 July 1935. The tips of the 3rd leaf were beginning to yellow and in many cases all the lower leaves had changed colour. Random samples of about 60 leaf strips were analysed initially, and after 24, 48, 72, 96, 120 and 144 hr.

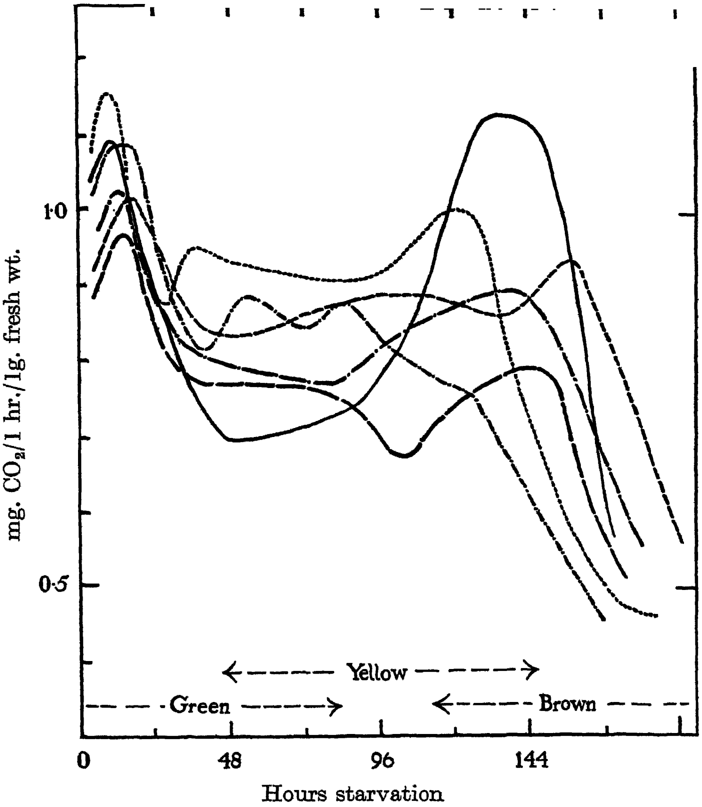


Fig. 1—Rate of carbon dioxide production during starvation: mean curves from Exps. VI–XI; Exp. VI ——; Exp. VII —·—; Exp. VIII -----; Exp. IX – – –; Exp. X — —; Exp. XI ·····.

a—Rate of Carbon Dioxide Production during Starvation

The results of measurement of the rate of carbon dioxide production in the different experiments are summarized together in fig. 1, all calculated to the basis of mg. CO_2/1 hr./1 g. fresh weight. Each curve shown represents the mean derived from separate samples used for carbon dioxide estimation, their number varying from twelve in Exp. VI to three samples in Exps. X and XI. The agreement between the different samples in the same experiment was nearly always within 5 % of the arithmetic mean, while the curves representing the drift during progressive starvation could be readily smoothed.

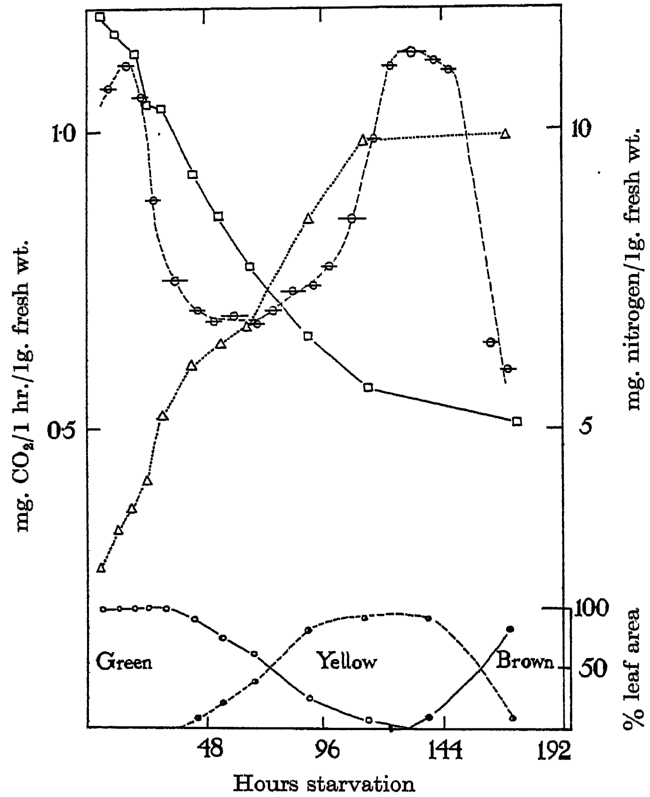


FIG. 2—Changes of total insoluble and total soluble nitrogen during starvation (Exp. VI) and mean rate of carbon dioxide production. Yellowing and browning of the leaves (as % area curves) shown for comparison. CO_2 production -⊖-; total insoluble nitrogen -□-; total soluble nitrogen - - -△- - -; % area, green -○-; % area, yellow - - -◑- - -; % area, brown -●-.

The rate of carbon dioxide production during starvation varied in a manner quite similar to that described in an earlier paper (Yemm 1935). The four conspicuous phases recognized there were always obvious—a short initial rise followed by a rapid fall in rate being observed in all experiments during the first 30–40 hr. Subsequently, during yellowing, the curves have a more variable form: this may be in part owing to the different cultural history of the leaves used for the separate experiments, and here the metabolic age of the leaf at the time of isolation seems to be an important factor. A rapid fall in the rate of carbon dioxide production intervened after from 120 to 150 hr. starvation, and was associated with browning and general disintegration of the leaves.

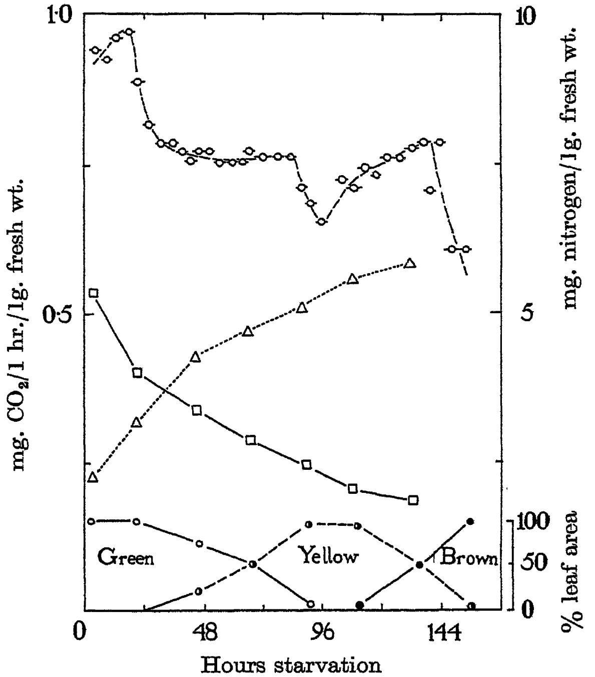


FIG. 3—Changes of total insoluble and total soluble nitrogen during starvation (Exp. X) and mean rate of carbon dioxide production. Yellowing and browning of the leaves shown for comparison. CO$_2$ production –○–; total insoluble nitrogen –□–; total soluble nitrogen - - -△- - -; % area, green –○–; % area, yellow - -◐- -; % area, brown –●–.

b—Changes of Total Insoluble and Soluble Nitrogen during Starvation

The estimations of the water soluble and insoluble nitrogenous compounds have shown that an increase of the soluble at the expense of the insoluble fraction occurred from the earliest stages of starvation. Considerable variation has been observed in the magnitude of the changes: the relevant data from two extreme cases, Exps. VI and X, are summarized in figs. 2 and 3. There was a clear indication in these and other experiments that the rate of breakdown of complex insoluble nitrogenous compounds was greatest during the earlier stages and became slower after considerable starvation.

It is unlikely that the error owing to the sampling method involved in these experiments was serious; from estimations of total nitrogen content of the different samples values for the probable error were calculated from the usual formula for separate experiments. For reasons to be given later only samples analysed before any considerable browning had occurred were used for these calculations, which are summarized in Table XII.

TABLE XII—"PROBABLE ERROR" OF TOTAL NITROGEN ESTIMATION IN SAMPLES OF EXPS. VI–XI

Exp. no.	No. of sample	Mean total nitrogen content (mg.)	Probable error ± %
VI	11	15·1	1·6
VII	5	11·6	1·3
VIII	6	7·6	2·0
IX	6	7·1	2·4
X	7	7·5	1·6
XI	6	8·2	2·2

Similar calculations for rate of carbon dioxide production of the samples gave values of ± 3–5 % for the probable error.

c—Changes of Amino, Amide and Ammonia Fractions during Starvation

Typical results of different series of analyses are summarized in figs. 4 and 5 (Exps. VII and XI), together with the appropriate carbon dioxide records for purposes of comparison. Every experiment has shown that during approximately the first 24 hr. of starvation the only considerable change involved the amino and unstable amide fractions; both of these rise sharply, so that their concentration was sometimes more than doubled over this initial phase. Over the same period only small fluctuations of stable amide and ammonia fractions were observed in all the experiments with barley leaves detached and starved under these conditions.

At about the 24th hour of starvation the stable amide started to increase, and rose to a high value during yellowing, while free ammonia accumulated slowly after about 48 hr. A final phase was characterized by the rapid formation of ammonia and a concurrent decrease of all the other fractions. Some experiments in which the gas stream was passed from the plant chamber through dilute sulphuric acid have shown that considerable quantities of ammonia were given off by the tissues after browning and

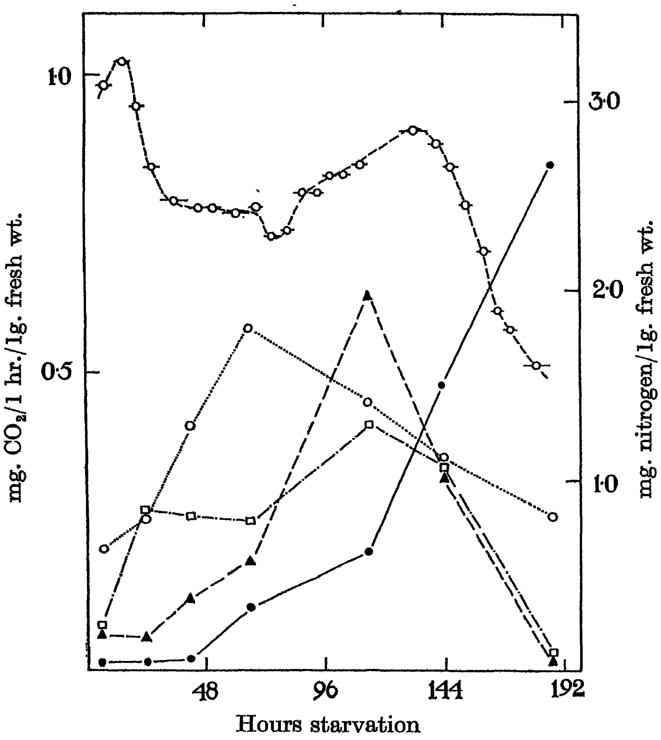


FIG. 4—Changes of amino, amide and ammonia fractions during starvation (Exp. VII) and mean rate of carbon dioxide production. CO$_2$ production –○–; amino nitrogen fraction - - -○- - -; 2 × unstable amide nitrogen —·—□—·—; 2 × stable amide nitrogen ——▲——; ammonia nitrogen ——●——.

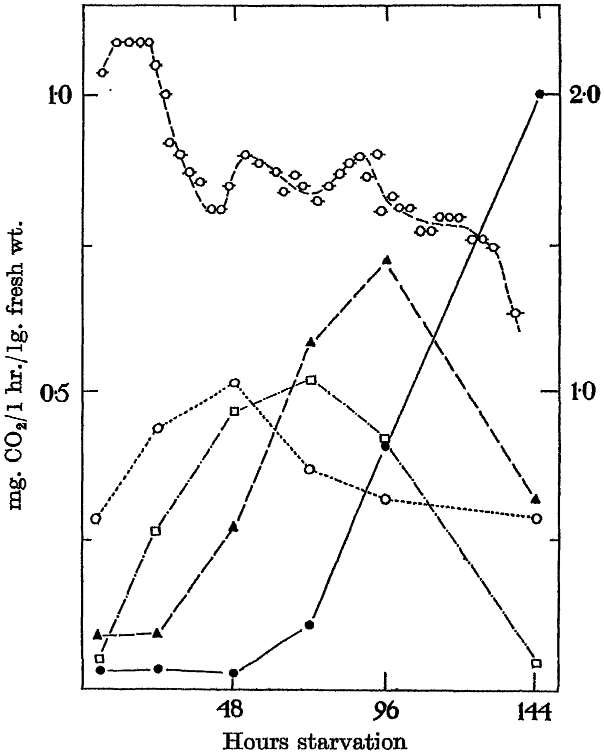


FIG. 5—Changes of amino, amide and ammonia fractions during starvation (Exp. XI), and mean rate of carbon dioxide production. CO$_2$ production –○–; amino nitrogen fraction - - -○- - -; 2 × unstable amide nitrogen —·—□—·—; 2 × stable amide nitrogen – –▲– –; ammonia nitrogen ——●——.

disintegration. Furthermore, the nitrogenous analyses of such samples gave values of the total nitrogen content which were significantly lower than the samples analysed before browning. So far no attempt has been made to determine the ammonia which is given off, and it follows that the estimates obtained by analysis of brown leaves did not give an estimate of the total ammonia formed. From about the 120th hour onwards they are in all probability too low owing to the escape of ammonia. In the separate experiments there was a correlation in time between the rapid rise in concentration of ammonia and the final fall in the rate of carbon dioxide production: with leaves that showed a later fall in rate of carbon dioxide production after 150–160 hr. starvation (Exps. VI–XI) the increase in ammonia content was later than that observed when the fall was earlier (Exps. VIII and XI). An attempt has been made to discover whether the accumulation of ammonia was a cause or an effect of the general disintegration of the cells during browning. This type of injury was first apparent in most experiments at the cut ends of the leaf after about 90–100 hr. starvation. At first it spreads only slowly, and the possibility existed that the slow rise in ammonia content observed at this phase might be caused solely by the breakdown of the cut cells by bacterial or autolytic means. Therefore, in one experiment the cut surfaces were always removed before the leaves were analysed, and it was found that during the first 100 hr. the ammonia content of the yellowing leaves rose to a value over ten times as high as that of the initial samples. In consequence it seems clear that a considerable accumulation of ammonia occurred in the yellowing leaves, irrespective of the surfaces injured by cutting.

Although the magnitude of the changes and their relation to the starvation time scale has varied somewhat, the relative times and general characteristics of the fluctuations in the different amino, amide and ammonia fractions have been essentially similar with all the barley leaves detached and starved under these conditions. The contrasting behaviour of the two amide fractions is of considerable interest: the collected results from the six experiments are shown in figs. 6 and 7.

The unstable amide fraction increased in a linear manner to 10–20 times the initial value during the first phase of starvation up to 30–48 hr., and afterwards there was considerable variation before the final decreases took place. As distinct from this the stable amide was nearly constant during a corresponding initial period, and only after starvation for longer than 24–48 hr. were significant increases detectable. Less variation was apparent in the form of the curves representing the fluctuations of stable amides; in all cases they show a maximum value at about the 120th hour, and then a rapid exhaustion, so that at the end of the experiments amides were present only in small quantities. In all experiments the rapid reduction in amide content was closely correlated with the accumulation of ammonia.

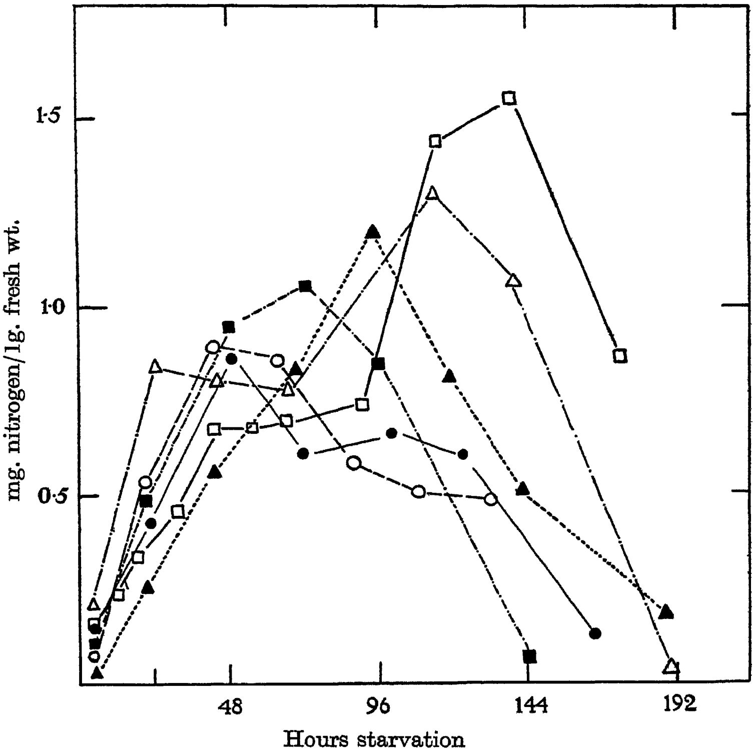


Fig. 6—Change of unstable amide nitrogen fraction during starvation: collected results of Exps. VI–XI. Exp. VI —□—; Exp. VII —·—△—·—; Exp. VIII - - -▲- - -; Exp. IX —●—; Exp. X - -○- -; Exp. XI —·—■—·—.

d—Changes of Soluble Nitrogen other than Amino, Amide or Ammonia Nitrogen

The separate fractions into which the soluble nitrogen was resolved accounted only in part for the total nitrogen present in the extracts. The remaining fraction, the chemical identity of which is uncertain, will be described as "Other soluble nitrogen", being determined by difference from the total soluble nitrogen and that accounted for as amino, amide or ammonia nitrogen. It has been found that during starvation the other soluble nitrogen always increased to some extent and then diminished in amount. As with the other fractions a considerable variation was observed in the magnitude of the changes, and extreme results, from Exps. VI and XI, are summarized in figs. 8 and 9. The total nitrogen

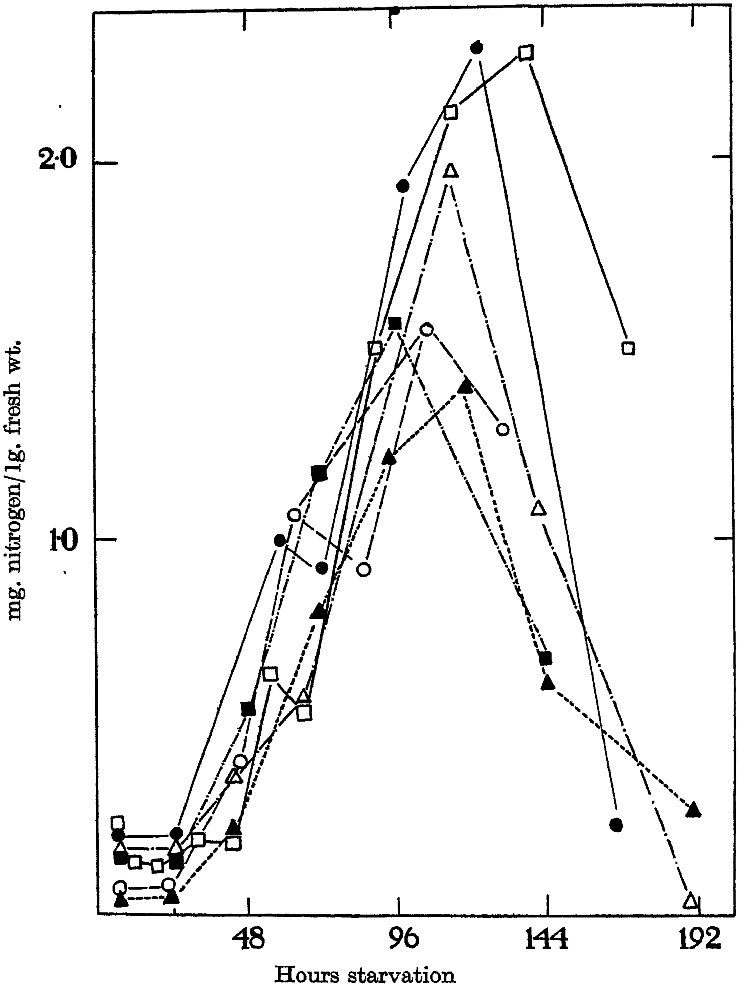


Fig. 7—Changes of stable amide nitrogen fraction during starvation: collected results of Exps. VI–XI. Exp. VI —□—; Exp. VII —·—△—·—; Exp. VIII - - -▲- - -; Exp. IX—●—; Exp. X - -○- -; Exp. XI —·—■—·—.

accounted for in the separate fractions increased in an almost linear manner until the final stages, when a decrease was observed: the preceding description makes it clear that this was in all probability owing to the escape of ammonia from the tissues.

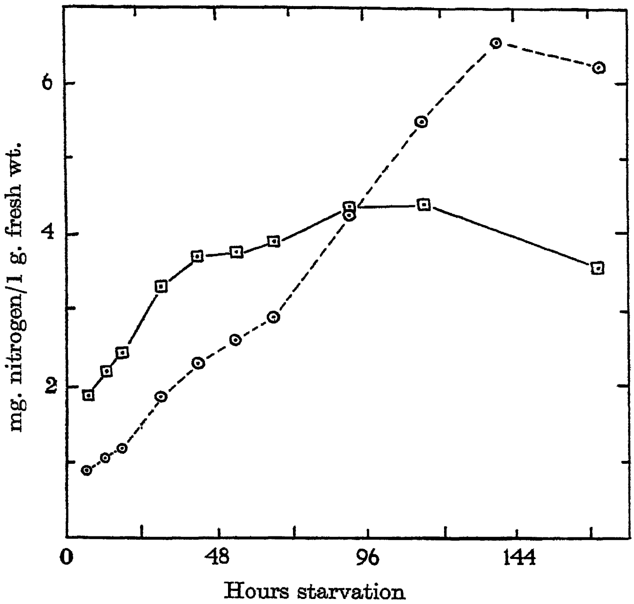


FIG. 8—Changes of soluble nitrogen other than amino, amide and ammonia nitrogen (Exp. VI). Other nitrogen —⊡—; amino, amide and ammonia nitrogen - -⊙- -.

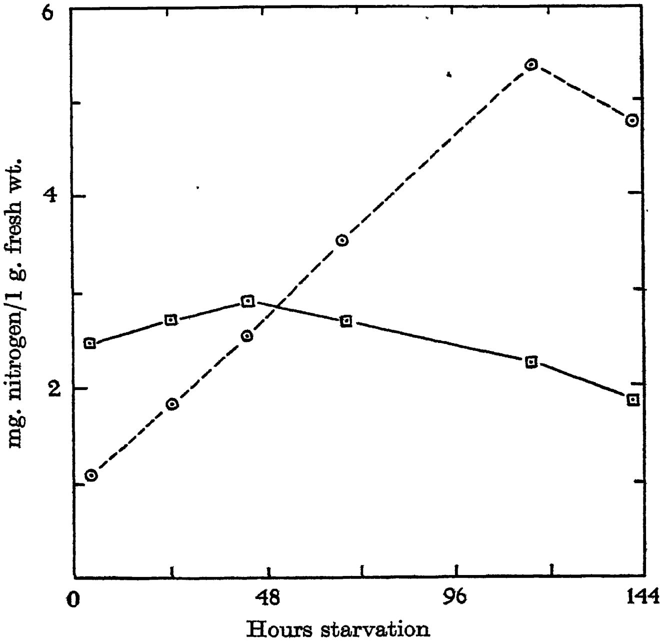


FIG. 9—Changes of soluble nitrogen other than amino, amide and ammonia nitrogen (Exp. XI). Other nitrogen —⊡—; amino, amide and ammonia nitrogen - -⊙- -.

DISCUSSION

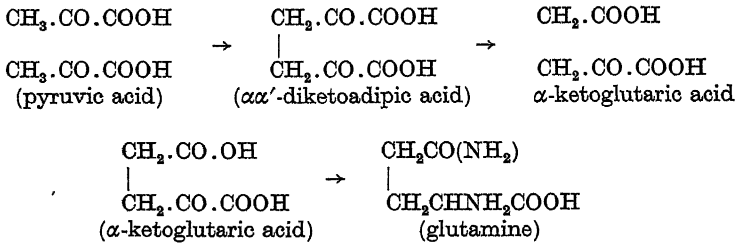
There is every indication that a comparison between the present results and the earlier ones concerned with carbohydrate exhaustion is justified. The measurements of carbon dioxide production show that many of the outstanding features of the other experiments are reproduced, and estimates of total soluble carbohydrates in an experiment parallel to Nos. X and XI of this series had results very similar to the experiments of 1932–3. It seems safe to assume that in all the experiments just described the initial phase, 0–24 hr., of respiration was associated with a rapid oxidative breakdown of normal carbohydrate metabolites, while later, during the yellowing phase, most of the carbon dioxide comes from oxidation of other substrates.

The analyses show that the breakdown of complex insoluble nitrogenous constituents begins at the very earliest stages, and it is clear that this is primarily a result of protein hydrolysis. The possibility exists that other insoluble nitrogenous compounds, such as chlorophyll, also break down, but all the evidence available suggests that this is relatively unimportant: chlorophyll, for example, only accounts for 2–3 % of the total nitrogen of tobacco leaves as shown by Vickery and others (1933). That the hydrolysis of proteins does occur is also shown by the increase of amino and amide fractions, although this increase does not account quantitatively for the loss of protein—other unidentified soluble nitrogenous compounds accumulate concurrently. A complex series of linked reactions undoubtedly intervenes between the hydrolysis of protein and the appearance of the simplest products; and any difference in the relative rates of these reactions may lead to an accumulation of peptides or other intermediate compounds. It is possible that the changes in the "other soluble nitrogen" fraction may be caused in part by such variations, although a definite answer can be provided only by further experiments.

The rate of breakdown of protein, as indicated by the decrease of insoluble nitrogen, seems to be greatest during the early stages of starvation; no true sparing of protein by carbohydrate can be detected, since during this initial phase the carbohydrate concentration is at its highest. A continuous hydrolysis of protein seems rather to be part of the normal mechanism whereby proteins, synthesized in the mature leaf, are translocated to other parts of the plant. Chibnall (1924*a*) and Maskell and Mason (1929) both detected a significant diurnal variation in the nitrogen content of normal attached leaves, and it is probable that there is a continuous hydrolysis, masked during the day by synthetic activity. In mature leaves isolated under starvation conditions it is likely that catabolic processes predominate, and the rapid accumulation of soluble nitrogenous compounds is the result.

The progressive decrease of protein nitrogen during leaf starvation has been observed quantitatively by many workers, Chibnall (1924*b*), Mothes (1926), Vickery and others (1933), while Meyer (1918) has drawn attention to shrinkage of the chloroplast during yellowing. Although Schumacher (1929) found that changes of protein content may occur without any detectable alteration in the size of the chloroplast, it is likely that much of the protein in the leaf cells is associated with the plastids. As shown by the present results the completion of yellowing corresponds with a considerable reduction in the rate of protein hydrolysis, similar to that observed by Vickery and others (1933) with tobacco leaves. It follows that this may be caused by the exhaustion of the proteins associated with the chloroplast, the rest of the complex nitrogenous substances in the cells being more resistant to hydrolysis. A further possibility is that the slower breakdown in the later stages may be caused by the inactivation of proteolytic enzymes by ammonia or other toxic products.

The changes of the simpler soluble nitrogenous compounds during starvation is of considerable interest, since this gives some insight into the further transformations of the products of protein hydrolysis. The changes during the first phase (0–24 hr.) are a conspicuous increase in the amino and unstable amide nitrogen fractions, and it is very probable that this is caused by the accumulation of amino acids, glutamine and peptides. In comparison with the other experiments of 1932–3, the simplest interpretation is that such compounds arise directly by hydrolysis of the leaf proteins, and are not at first seriously involved in secondary oxidative changes. Many workers have observed increases of amino nitrogen during leaf starvation and have attributed it to the hydrolysis of proteins, but the significance of the unstable amide glutamine or its peptides is less clear. One of the characteristics of many plant proteins, especially those of cereals, is their high glutamic acid and ammonia content, as determined by acid hydrolysis. This is also true of leaf proteins from the related grass *Dactylis glomerata* (Millar 1936). Damodaran, Jaabach and Chibnall (1932) have shown that such proteins yield glutamine and glutamyl peptides on enzyme hydrolysis *in vitro*, and conclude that glutamic acid is present in the protein molecule in the amide form. It follows, therefore, that unstable amide may be an important product of protein hydrolysis in living tissues, although there is little other direct evidence; Chibnall and Greenhill (1934) have recognized this possibility. In detached tobacco leaves Vickery and others (1933) detected the presence of an unstable amide fraction, and have since isolated pure glutamine and asparagine from the leaves (Vickery and Pucher 1936). While a difference in the conditions under which the detached leaves were kept makes direct comparison difficult, the unstable amide appears to change differently in tobacco leaves, only accumulating when the leaves were highly starved and yellow. A difference in hydrolysis or constitution of the proteins may be responsible, but it is possible that under some conditions glutamine is synthesized in plants from carbohydrates and ammonia by a series of reactions similar to those suggested by Weil-Malherbe (1936):



$$\begin{array}{c}\mathrm{CH_3.CO.COOH}\\ \\ \mathrm{CH_3.CO.COOH}\\ \text{(pyruvic acid)}\end{array} \rightarrow \begin{array}{c}\mathrm{CH_2.CO.COOH}\\ | \\ \mathrm{CH_2.CO.COOH}\\ (\alpha\alpha'\text{-diketoadipic acid})\end{array} \rightarrow \begin{array}{c}\mathrm{CH_2.COOH}\\ \\ \mathrm{CH_2.CO.COOH}\\ \alpha\text{-ketoglutaric acid}\end{array}$$

$$\begin{array}{c}\mathrm{CH_2.CO.OH}\\ | \\ \mathrm{CH_2.CO.COOH}\\ (\alpha\text{-ketoglutaric acid})\end{array} \rightarrow \begin{array}{c}\mathrm{CH_2CO(NH_2)}\\ | \\ \mathrm{CH_2CHNH_2COOH}\\ \text{(glutamine)}\end{array}$$

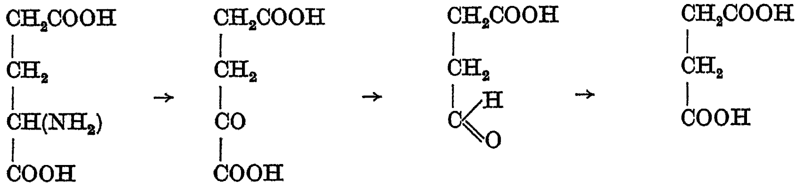
If the glutamine were formed in barley leaves by reactions such as these, the effect on the total gaseous exchange would be scarcely beyond the limit of accuracy with which the respiratory quotient was determined, and at present there is no means of deciding whether this alternative fate of metabolized carbohydrate is important. In consequence the evidence that unstable amides arise solely from protein hydrolysis is not conclusive. However, such a hypothesis gives a consistent interpretation of both the experiments described here and others in which the leaves were detached and starved under different conditions. More information is desirable, and experiments are being planned with this end in view.

During the next phase, following the first 24 hr. starvation, a conspicuous change in the nitrogenous constituents was always detected; the stable amide fraction began to accumulate, followed a little later by a significant increase in the ammonia content of the yellowing leaves. Although other nitrogenous compounds may be included in the stable amide fraction it seems certain that asparagine is the main constituent. The interrelationship of asparagine and ammonia with protein metabolism has been the subject of much investigation and controversy. At present the most generally favoured view, originating with Boussingault (1864), is that in starved tissues both of these substances may arise during the secondary decomposition of proteins, the asparagine formation being regarded as a secondary synthesis, whereby the toxic effects of ammonia, resulting from deamination, is prevented; relevant observations with both seedlings and leaves have been reviewed by Murneck (1935). This hypothesis is in accord with the observations recorded here, and it seems that a correct interpretation may be made along these lines: the time at which the stable amide begins to accumulate corresponds closely with that at which the breakdown of protein appeared to be important in carbon dioxide production, as deduced from both carbohydrate analyses and measurements of the respiratory quotient.

Damodaran (1932) has shown that asparagine is formed in the enzyme hydrolysis of some proteins *in vitro*, and it follows that some stable amide may arise in this way in starving leaves. However, the aspartic acid content of most plant proteins, as indicated by acid hydrolysis, is much lower than their glutamic acid content, and this is true also of the leaf protein studied by Millar (1936). The possibility that asparagine is always a primary product of protein hydrolysis was eliminated by Schultze and Castoro (1903) in the case of lupin seedlings, since the yield of asparagine was much greater than could be accounted for by the aspartic acid content of the proteins originally present.

It is generally held that asparagine is formed in plants by a synthesis from ammonia and 4-C acids, although the infiltration experiments of Mothes (1933) have been criticized by Schwab (1935). The ammonia in starved leaves presumably arises from active deamination, while it is becoming increasingly clear that the production of 4-C acids may be intimately connected with carbohydrate metabolism (Bennet Clark 1933; Bennet Clark and Woodruff 1935). The period of rapid asparagine accumulation in starving barley leaves corresponds with that at which a relatively low steady rate of carbohydrate loss occurs from about the 30th to the 100th hour, while the rapid formation of ammonia occurs when the exhaustion of normal carbohydrates is almost complete. The failure in the ammonia-binding mechanism may, therefore, be attributed to extreme carbohydrate starvation. A similar view of the effect of carbohydrate content on the fate of ammonia in plant tissues is expressed by Prianischnikov (1924), who found that ammonia presented to seedlings is stored as amide nitrogen if a moderate supply of carbohydrate is available, but as unchanged ammonia in conditions of extreme carbohydrate shortage. Prianischnikov's experiments have been criticized by Buckhart (1934), but it appears that ammonia accumulates only in tissues with a very low concentration of reducing sugar.

Another possible way, other than by the production of 4-C acids in glycolysis, in which the presence of carbohydrate may be effective in controlling the synthesis of stable amides must be noticed. An analogous system to that in yeast, investigated by Ehrlich (1909) and Neuberg and Ringer (1918), may be of general biological importance; the following series of reactions seems to be well established whereby glutamic acid is converted to succinic acid:



Glutamic acid → (α-ketoglutaric acid) → (succinic semialdehyde) → (succinic acid)

Succinic acid is produced only in presence of sugar, and since Krebs (1935) has shown that α-ketoglutaric acid is actively decomposed by yeast and no additive effect occurs with glucose, it is possible that a sparing of the deaminized residue by the carbohydrate is important. Although the physiological significance of such reactions in the metabolism of the different organisms is distinct, there is considerable evidence of similarity in other important catabolic mechanisms. A similar series of reactions to those indicated above may, therefore, be effective in transforming glutamine by way of the 4-C acids or acid amides to asparagine, the presence of sugar being decisive in preventing further oxidation of the deaminized residues. That glutamine may be converted to asparagine in starving barley leaves is suggested by some of the experiments described here: the rapid rise in unstable amide which occurs at first is interrupted at a time when the accumulation of stable amide starts. If the total amide content is considered an almost linear increase is apparent up to the time of rapid ammonia formation. It is of course possible that other mechanisms may account for this relationship.

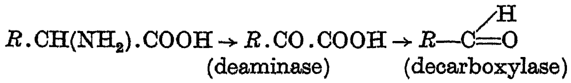
The precise connexion between the different nitrogenous transformations and the gaseous exchanges of the leaves is very difficult to decide. The analytical methods at present give little information concerning the nature and fate of the carbon residues resulting from deamination, and these are presumably important in subsequent oxidation and production of carbon dioxide. It is significant that very little change in the ammonia content of the tissues occurs during the early stages when a respiratory quotient of about unity was observed. As shown by Needham (1932) a quotient of this order may result if protein decomposition occurs with ammonia as an end product; this possibility is therefore eliminated and the conclusion that most of the carbon dioxide originates at first from the oxidation of carbohydrate is strengthened.

Since the end products are different at different stages of starvation, it is probable that this would be reflected in the respiratory quotient characteristic of protein breakdown. If it is assumed that asparagine and ammonia together with carbon dioxide and water represent the final products, a calculation shows that the formation of asparagine implies a quotient of about 0·7, and ammonia one close to unity. From earlier carbohydrate analyses a calculation of the respiratory quotient made on the assumption that protein catabolism involved a steady value of 0·8, showed a general similarity with the respiratory quotient observed experimentally. It is probable that some of the minor differences between the calculated and observed values may be explained on the basis indicated above; during the early stages of yellowing the calculated value is predominantly higher than that observed, and here the analyses recorded suggest that asparagine may be an important end product. Later, just before and during browning, the calculated value is lower; at this stage it is probable that ammonia is the chief end product, and the quotient characteristic of protein breakdown should be nearer unity than 0·8.

From these results it seems clear that the fairly steady value of the respiratory quotient at a value just higher than 0·8 between 48 and 100 hr. of starvation is in reality complex. The low value of about 0·7 characteristic of protein breakdown to asparagine may not be realized, since at first it is accompanied by carbohydrate breakdown, and later by protein catabolism to ammonia. This smoothing effect is very probably owing to the method of using relatively large numbers of leaves for such experiments. There are clear indications not only that different leaves but also different cells of the same leaf may react to starvation on a slightly different time scale, and therefore gaseous and biochemical changes as estimated by analysis of a sample represent the mean of a number of different systems. It is possible, therefore, that the form of the transition from one type of mechanism to another may be a result of the relative number of cells behaving at the different extremes rather than a characteristic of the cell mechanism. Such a qualification is necessary in interpreting results such as mine, when the heterogeneity of the leaves is not more fully defined. While the changes are qualitatively characteristic of the cells themselves they are most probably not quantitatively representative of the cell mechanism. Considered from this aspect a general summary of the changes, which have been described here and elsewhere, and their probable connexion with the respiratory quotient of the starving leaves is given on p. 270.

Period no.	Approx. hours	Catabolic changes	Respiratory quotient
1	0 → 24	Carbohydrate	1·0
2	24 → 48	Carbohydrate Nitrogenous → amide	1·0 → 0·8
3	48 → 100	Carbohydrate Nitrogenous → amide Nitrogenous → ammonia	0·8
4	100 →	Nitrogenous → ammonia	0·8 → 0·9

The absence of a suitable nitrogenous substrate does not seem to be primarily responsible for the preferential metabolism of carbohydrate, detected both in these experiments and those of Vickery and others (1933). The results suggest that the mechanism of the subsequent oxidation does not involve an entirely different enzyme system for each substrate: it is probable that there is (at least) one component in common, possibly that concerned with the activation of molecular oxygen. There is considerable evidence that one of the first steps in the aerobic breakdown of amino acids involves an oxidative deamination to the corresponding α-keto-acid, which may then be decarboxylated in the presence of decarboxylase.



$$R.\mathrm{CH(NH_2).COOH} \xrightarrow[\text{(deaminase)}]{} R.\mathrm{CO.COOH} \xrightarrow[\text{(decarboxylase)}]{} R\text{—}\overset{\diagup \mathrm{H}}{\mathrm{C}}{=}\mathrm{O}$$

At either of these stages competition with more labile products of glycolysis may be effective in preventing the breakdown of nitrogenous constituents until sugar exhaustion has occurred.

The rapid accumulation of ammonia in the tissues may be an important factor in the final disorganization of the protoplast, since the toxicity of this end product is well known. It is possible that infection by micro-organisms may have contributed to ammonia production in the final stages, since no elaborate precautions were taken to ensure sterility. A rapid rise in rate of carbon dioxide was not observed at the end, such as that detected by Godwin and Bishop (1927), and fungal and bacterial invasion may be checked by the relatively large amount of ammonia present. In some experiments a loose hyphal weft was observed at the end of browning, but this appeared to be mainly superficial. A source of error is apparent in the gasometric estimations when the rapid rise in ammonia content occurs: carbon dioxide may be retained in the form of ammonium carbonate. This would lead to a false depression of the CO_2/O_2 ratio, so that the true oxidation quotient may be higher than that observed in the later stages; and similarly the very rapid fall in rate of carbon production may be in part caused by the retention of carbon dioxide in the tissues.

It is not proposed to give a detailed description here of the individual features of the separate experiments; an attempt will be made later to correlate differences in the carbon dioxide records with fluctuations of some of the nitrogenous constituents, when other relevant experiments have been described. It is sufficient to indicate that unstable amides, either glutamine or its peptides, seem to be of particular interest in this respect; the unique properties of glutamic acid and probably glutamine in contact with a wide range of biologically important systems is becoming increasingly apparent (Thunberg 1920; Harrison 1935; Needham 1930; Krebs 1935; and Weil-Malherbe 1936). In many tissues that are primarily concerned with carbohydrate metabolism, glutamic acid, as distinct from almost all other amino acids, may be rapidly oxidized to carbon dioxide. The intricate changes in starving leaves present special difficulty in that other concurrent changes may have a masking effect, but there is some evidence that glutamine or its peptides may be important nitrogenous metabolites influencing the rate of carbon dioxide production after the first phase of carbohydrate exhaustion.

The sequence of changes in starving leaves, implying a metabolism of carbohydrate followed by extensive utilization of a nitrogenous substrate, finds a parallel in many living tissues. It is highly suggestive of a greater similarity of catabolic mechanism than might at first be suspected, although the physiological significance of the changes may be distinct. In the present experiments it is likely that the rapid catabolism of nitrogenous substances in the respiration of the leaf is artificial in the sense that it is a result of the special experimental conditions: the view, originally expressed by Pfeffer (1897), may apply in that under conditions of starvation substances not normally used may be drawn into the metabolism. Further indications of this have already been obtained with leaves starved while still attached to the plant. It is highly probable that under normal circumstances the respiration of higher plants is dominated by carbohydrate changes and that proteins are relatively less important with the possible exception of some germinating seeds.

The author is indebted to Professor Tansley and Dr. W. O. James for the facilities which made this work possible, and also to Mr. J. L. Harley for his assistance in the collection and preparation of leaf material.

SUMMARY

The methods of estimation and changes of some of the important nitrogenous constituents in starving barley leaves have been described, and the

results discussed in relation to earlier experiments on carbohydrate exhaustion. Some of the important biochemical mechanisms which may be involved in the co-ordination of the carbohydrate and protein metabolism have been indicated.

An interpretation of the results, consistent with all the data at present available, may be summarized as follows:

1. Hydrolysis of proteins is continuous in the detached starving leaves, and leads at first to the accumulation of soluble nitrogenous compounds most probably identified as amino acids, glutamine, or peptides.

2. When a fall in carbohydrate concentration occurs the primary products of hydrolysis are further decomposed, an important nitrogenous end product being at first stable amide, probably asparagine.

3. Extreme carbohydrate exhaustion is associated with a rapid accumulation of ammonia, which may be a proximate cause of the death of the tissues.

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The Isolation and some Properties of Liquid Crystalline Substances from Solanaceous Plants infected with Three Strains of Tobacco Mosaic Virus

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(*Communicated by Sir Frederick Gowland Hopkins, O.M., F.R.S.—Received* 17 *March* 1937)

[Plates 12, 13]

Tobacco mosaic was the first disease shown to be caused by a filter-passing virus, and Beijerinck (1898) suggested as its cause a "contagium vivum fluidum". Since then many other theories have been advanced, but there has been little positive evidence to indicate whether the virus more nearly resembled organisms such as small bacteria or chemical molecules such as the larger proteins. Recently, however, Stanley has isolated from tobacco (1936*a*) and tomato (Stanley and Loring 1936) plants suffering from mosaic a protein which he describes as crystalline and as possessing the properties of the virus. When susceptible plants were inoculated with this protein at a dilution of 10^{-9} they developed typical symptoms of the disease. The protein was obtained from infective sap by repeated precipitation with 40% saturated ammonium sulphate solution, and by adsorption on and washing from celite. The "crystals" described by Stanley were small needles produced by precipitation with acid ammonium sulphate.

A number of statements in Stanley's earlier paper (1935*a*), more especially those dealing with the nitrogen content and the serological activity, made us doubt the purity of his product, and preliminary experiments with methods similar to those used for the preparation of suspensions of potato virus "*X*" (Bawden and Pirie 1936) gave us products with much higher precipitation end-points with antisera than those claimed by Stanley. We have now exchanged material with Dr. Stanley and find no gross differences in the activities of our respective products. We have found, however, that by further purification the protein in neutral solution can be obtained in liquid crystalline states. Also, as will be shown later, there are considerable differences in the chemical descriptions given of the virus protein; some of these differences have already been resolved, and others presumably will be by future work.

VIRUS STRAINS USED

Tobacco mosaic virus is known to exist in a number of strains which possess similar properties *in vitro*, e.g. resistance to ageing, temperature, etc., yet differ from one another in the ease with which they can be transmitted to different hosts and in the type and severity of symptoms they cause in infected plants. We have worked with three such strains and have isolated liquid crystalline proteins from various plants infected with each. Irrespective of the host plant from which it was obtained, inoculation of the isolated protein to susceptible plants caused a disease characteristic of the particular virus strain used, and from such infected plants more of the same protein could be isolated.

The three strains used were a mild strain of the common tobacco mosaic virus, aucuba mosaic virus and Enation mosaic virus. The first was derived from a single local lesion on *Nicotiana glutinosa* produced by inoculating with diluted sap from a plant infected with Johnson's tobacco virus 1. The second was kindly provided by Dr. F. M. L. Sheffield and the third by Dr. G. C. Ainsworth. The symptoms produced by each strain vary with the age and conditions under which the host plants are grown. Those briefly described below are characteristic of plants growing under good light conditions in an insect-proof glasshouse at an average temperature of 70° F.

The strain of tobacco mosaic virus used causes in tobacco (vars. White Burley and Turkish) a preliminary clearing of the veins, followed by a general green-type mottling, with a few darker green blotches around the veins and occasional small yellow flecks. In tomato (var. Kondine red) it causes a general faint mottle and no deformation of the foliage; in old plants symptoms are often completely masked, so that the hosts behave as carriers.

Symptoms of aucuba mosaic have been described in detail by Henderson Smith (1928). In tomato and *Solanum nodiflorum*, the hosts used most in this work, they consist of a brilliant yellow mottle. Tobacco also was used but was not a convenient host because of the difficulty in getting uniform results. On large tobacco plants no systemic infection was obtained: the inoculated leaves developed severe necrotic local lesions, but the uninoculated leaves remained healthy. Systemic infections were usually obtained in small seedlings, and these showed a bright yellow mottle, but they were so crippled by the disease that they yielded only small amounts of sap.

The hosts used for Enation mosaic virus were tobacco and tomato. Inoculated tobacco plants developed severe necrotic local lesions similar to those caused by aucuba mosaic virus, and, unless the plants were small, usually did not all become systemically infected. Those that did become infected showed symptoms similar to, but rather more severe than, the symptoms of plants infected with tobacco mosaic virus. On tomato, however, the disease caused by this strain is quite characteristic. Infected tomato plants developed a general mottle. The leaves were severely deformed and much dwarfed, often consisting of little more than the main veins, so that the picture obtained was typical "Fern-leaf". The under-surfaces of the leaves frequently developed leafy outgrowths, the so-called enations, which vary in size from minute ridges to large leafy frills (Ainsworth 1935).

Although the symptoms produced by the three virus strains have remained reasonably constant, it is not thought that each source necessarily contains only one strain. Indeed, it is more probable either that the sources are not pure or that they are continually mutating, for, from the yellow flecks appearing in the tobacco mosaic plants, material for an inoculum could be obtained that caused a general, bright yellow mottle in tobacco. This phenomenon has been fully described by Jensen (1933).

All three virus strains produce necrotic local lesions in *Nicotiana glutinosa*. They are serologically related, and all three are neutralized *in vitro* by, and precipitate with, the serum of rabbits immunized against the sap of plants infected with tobacco mosaic virus. Further, tomato plants infected with tobacco mosaic virus were found to be immune to further infection with either aucuba mosaic or Enation mosaic viruses.

Activity of the Purified Virus Preparations

Liquid crystalline proteins have been isolated from all the plants affected with the diseases described, and the kind of protein isolated was found to depend entirely on the virus strain and not at all on the host plant used. The activity of the proteins was tested in two ways: (1) for their infectivity, and (2) for their precipitation end-point with the serum of rabbits immunized by a course of intraperitoneal injections with crude sap from plants infected with the tobacco mosaic virus. As with potato virus "*X*" preparations, the two methods of measuring activity agreed quantitatively only when the preparations which were being compared had been treated similarly before they were tested (Bawden 1935).

Infectivity tests were made by the local-lesion method. At first both *N. glutinosa* and Golden Cluster beans were used, but as under the glasshouse conditions at Rothamsted the beans proved both less sensitive and more variable, in later work only *N. glutinosa* was used. The number of local lesions produced by the same purified virus preparation varied considerably with the age and condition of the host plant, and with the position of the inoculated leaves on the stem. In determining the effect of dilution in individual experiments the host variation was reduced to a minimum by arranging the inoculations in the form of Latin squares. Each dilution was inoculated once to each plant and once to a leaf occupying the same relative position on the stem. When two virus preparations were being compared over a range of dilutions the Latin square was split for half-leaf comparisons, solutions of the same concentration being inoculated to opposite halves of the same leaves. The inoculations were made with ground-glass spatulae, and the fluids were rubbed as evenly as possible over the leaves. In the infectivity tests dilutions were made in 0·1M phosphate buffer at *p*H 7, and in the serological tests in 0·85 NaCl solution.

The precipitation end-point determinations were made in 7 mm. thick-walled glass tubes, 1·0 c.c. of antiserum at a dilution of 1 in 50 being added to a series of tubes each containing 1·0 c.c. of protein solution at various concentrations. The tubes were immediately placed in a water-bath at 50° C. with fluid columns half-immersed to ensure complete mixing by convection currents. Readings were made after 24 hours, the first twelve being at 50° C. and the second at room temperature. The end-point was taken as the amount of protein in the tube containing the smallest precipitate visible to the eye.

The results of a few such tests on purified virus preparations before and after drying over P_2O_5 are given in Table I.

The infectivity of our preparations is of the same order as that obtained by Stanley (1936*a*) with tobacco mosaic virus, but the serological activity is from 50 to 100 times as great, for Stanley obtained precipitates with $1/10^5$ g. but not with $1/2 \times 10^5$ g. In a recent paper on aucuba mosaic virus (1937) Stanley states that "solutions containing slightly less than $1/10^6$ g./c.c. may give the precipitin test...but that at such high dilutions the determinations are not reproducible at will". Stanley does not give the methods used in his tests, and it is therefore difficult to account for these differences. Using the method described above, the serological titres of from $1/10^6$ to $1/10^7$ with our preparations are readily reproducible. With the more highly purified virus preparations to be described later precipitation end-points of $1/10^7$ are common, and repeated tests on the same preparation give quite constant results. The serological titre is a reliable index of the antigen content of a solution, for not only are the results obtained reproducible but the end-point is relatively independent of the concentration of antiserum used. All the tests reported in this paper were made with antiserum at a dilution of 1 : 50, but deviations from this figure affect only the position of the optimal precipitation point and have no appreciable effect on the precipitation end-point of a virus preparation.

TABLE I—ACTIVITY OF THE PURIFIED VIRUS PREPARATIONS, BEFORE AND AFTER DRYING OVER P_2O_5

Virus	Serological titre	Infectivity on *N. glutinosa* Average no. of lesions per half-leaf at various dilutions 10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Tobacco mosaic undried	$1/6 \times 10^6$	125	44	19	2·4	1·3	0·14	0
Tobacco mosaic dried	$1/2 \times 10^6$	103	22	9	1·5	0·3	0·14	0
Tobacco mosaic undried	$1/8 \times 10^6$	—	90	37	6	2	0·4	0·2
Tobacco mosaic dried	$1/4 \times 10^6$	—	56	29	3	0·3	0·14	0
Tobacco mosaic undried	$1/8 \times 10^6$	208	120	28	3	1	0·25	0·125
Aucuba mosaic undried	$1/10^7$	—	71	15	3·5	0·5	0·16	0
Aucuba mosaic dried	$1/4 \times 10^6$	—	42	6	1·3	0·16	0·16	0
Aucuba mosaic undried	$1/10^7$	105	70	17	3	0·2	0	—
Enation mosaic undried	$1/6 \times 10^6$	176	76	13	1·33	0·5	0·16	—
Enation mosaic dried	$1/2 \times 10^6$	104	47	9	0·66	0·66	0	—
Enation mosaic undried	$1/5 \times 10^6$	149	36	4	0·6	0·2	—	—

Footnote to Table I. The serological titre is the number of grams in 1 c.c. of a solution that gives a visible precipitate after 24 hr. with 1 c.c. of antiserum at 1 in 50. Dilutions in the infectivity tests are given as grams per c.c. The infectivity tests were arranged in the form of split Latin squares with from six to nine replications, i.e. 1 c.c. at each concentration was rubbed over from six to nine half-leaves.

The infectivity tests, however, give much less constant results. The number of local lesions obtained depends largely on the host plant, and can be altered by varying the conditions under which the plant is grown. Repeated tests on the same virus preparation have given infection end-points varying from $1/10^8$ to $1/10^{11}$. The relationship between dilution and infectivity is rarely a linear one, but the range over which the fall in the number of lesions is most nearly proportional to dilution is from $1/10^5$ to $1/10^8$.

No large differences between the activities of preparations of the different virus strains have been noticed, but accurate comparative infectivity experiments have not been made. All the precipitation end-point determinations were made with antiserum to tobacco mosaic virus, and with this the preparations of Enation mosaic virus have given slightly lower serological titres than have those of the other two strains.

Purified preparations of all three strains are antigenic, and antisera have been prepared against each. Each virus precipitates with and is neutralized *in vitro* by all three antisera. From the results of preliminary cross-absorption experiments, however, it seems probable that although the three are serologically related they are not antigenically identical, and that each contains specific as well as common antigens.

It is still not proved that the liquid crystalline proteins, which we shall describe, are the viruses, for it is not possible to state that our preparations contain only particles capable of infecting susceptible plants.The infectivity results show that solutions must contain about 10^{-9} g. of protein per c.c. to cause infection by the methods used. This weight of protein would represent a large number of virus particles. As the amount of virus required to produce a given number of local lesions varies with individual plants (and with the conditions under which they are grown) it is possible that a natural resistance of the plant has to be overcome before infection can occur, and a large number of particles may be necessary to do this. Also, the method of inoculation is relatively crude and there must be a large wastage of inoculum on the leaf surface. However, until infection can be obtained with much less protein, we are unable to see how conclusive evidence that the protein is virus can be obtained.

The serological results show that our preparations have precipitation end-points similar to those given by the more highly purified antigens; from the serologist's point of view, therefore, they may perhaps be regarded as relatively homogeneous. The purified virus preparations do not precipitate with the serum of rabbits immunized against either healthy tobacco sap or against sap of plants infected with viruses not related to tobacco mosaic virus. There is a great deal of circumstantial evidence indicating that the extra antigens in virus-infected plants may be the viruses themselves, but this again is not proved. There is a possibility that the antigens we have isolated are not the viruses but abnormal proteins produced as a result of the disease and contaminated with small amounts of virus. We consider this improbable, but there is no conclusive evidence against it. With potato virus "*X*" it has already been shown (Bawden 1935; Bawden, Pirie and Spooner 1936) that antigenicity can be dissociated from infectivity by chemical treatment, and Stanley (1936*b*) recently obtained similar results with tobacco mosaic virus. We have found with our preparations of tobacco mosaic viruses that severe treatments such as heat, strong acid or alkali destroy their infectivity, antigenicity and crystallinity, whereas less severe treatments, such as nitrous acid or subjection to X-rays, may destroy infectivity without affecting antigenicity or crystallinity.

The data from centrifuging experiments and X-ray measurements, which will be described later, suggest that our preparations consist solely of particles that are very similar to one another in size and shape. The chemical evidence shows that successive isolations of protein agree with each other closely, and that the chemical properties are not changed by further fractionation. It is reasonable to assume, therefore, that if all the particles in our preparations are not virus particles capable of infecting plants, they are at least similar to virus particles in their superficial properties. We have no evidence that virus activity can be dissociated from the isolated proteins, and for simplicity shall refer to them as virus. *We shall assume, however, that the reservations stated above are remembered when, in the remainder of this paper, we speak of our product as virus.*

PREPARATION

Infected leaves are minced in a meat mincer and the sap expressed in a hand press. The sap can usually be clarified by centrifuging immediately, but sometimes it clears more easily if allowed to stand overnight or if it is first adjusted to about *p*H 5·2. Alternatively, it may be clarified by filtration through celite or Kieselguhr. The procedure we adopt for the isolation varies somewhat according to the state of the sap, but the method given in detail below can be used successfully with all types of sap we have yet encountered. With sap from young plants which have not long been infected, however, it is possible to shorten the process considerably.

One volume of alcohol is added to the clarified sap, and the greyish brown precipitate is centrifuged off immediately. The precipitate contains all the virus: there is evidence, which we shall present later, that this precipitation leads to an irreversible aggregation of the virus particles. The final product, therefore, is not strictly in the same state as the virus in the plant sap. This aggregation, however, is at present unavoidable, for a similar aggregation follows precipitation with acid and with ammonium sulphate, and we have no other effective means of isolation.

Attempts to isolate the virus by high-speed centrifugation or by filtration on collodion membranes have not as yet given products with a serological activity comparable with that of the preparations described below.

The alcohol precipitate is suspended evenly in from five to six times its volume of water and again centrifuged. The first extract contains little or no virus and is discarded. The extraction of the precipitate is now repeated until a sample of the extracts no longer gives a precipitate with phthalate buffer at *p*H 3·3. From six to eight washings are usually necessary to remove all the virus from the chocolate-coloured residue. The collected supernatant fluids are now brought to about *p*H 3·3 by the addition of N/10 HCl. A white precipitate with a very characteristic satin-like sheen is produced, and this is centrifuged off. This precipitate is now suspended in water and dilute NaOH added to bring the *p*H to 7, when the fluid is again centrifuged until clear. The darkly coloured precipitate is discarded, and a third of a volume of saturated ammonium sulphate solution is added to the supernatant. This again produces a precipitate with a sheen and, after centrifuging, a yellow-coloured supernatant liquid which is discarded. The precipitate is taken up in water and again precipitated by adding a third of a volume of saturated ammonium sulphate solution.

The precipitate is now dissolved in water, using about 50 c.c. for the amount obtained from a litre of sap, and N/10 HCl is added to bring the *p*H to 3·3 (measured with bromophenol blue). This usually requires about 4–5 c.c. of acid. The precipitate is centrifuged off, resuspended in water and again centrifuged. The precipitate is now dissolved in N/50 NaOH, diluted to 50 c.c. and centrifuged if not quite clear. The neutral solution is now again brought to *p*H 3·3 and the precipitate centrifuged off. The precipitate is now relatively free from salts, and in this condition the material is soluble at *p*H 3·3, i.e. in N/200 HCl. 0·5 c.c. of N/20 HCl is therefore added to the precipitate and the mixture is stirred until it is homogeneous. When diluted to about 50 c.c. with water, a clear or slightly opalescent solution is obtained from which a little impurity can usually be removed by centrifuging. The virus itself, giving the characteristic satin-like sheen, can now be precipitated from the solution merely by adding 0·5 c.c. of 10 % NaCl solution. Alternatively, the virus can be precipitated from the salt-free solution by adding enough dilute NaOH to bring the *p*H to about 4. The precipitate obtained in this way, however, seems to be amorphous.

The salt-free precipitate is then dissolved in enough dilute NaOH to give a solution at about pH 6·5. Such a solution remains active for months when kept cold. If kept at room temperature it should be protected from evaporation, but it is unnecessary to adopt strict sterile precautions for periods of a few days, as bacteria and moulds do not grow at all readily in these solutions.

Solutions prepared in this way should be nearly colourless and slightly opalescent. Preparations of the three different strains show no consistent differences, although different batches of the same virus strain may vary considerably in appearance if they contain different amounts of impurities. The preparations, if not weaker than about 4 %, should separate into two layers after standing for some time. Each layer is liquid and contains virus, but the lower is both a stronger solution and also contains less impurity than the upper. This phenomenon and its application to further purification will be more fully described later.

Some preparations at this stage are still coloured, and on standing will not separate into layers. If the pigmentation is noticed early on in the preparation, or on a preliminary sample, a great improvement can be made by repeating the precipitation with alcohol on the eluate from the first alcohol precipitate. If, however, the virus has already been precipitated with acid it can no longer be precipitated with 50 % alcohol, for this precipitation depends on the presence in the fluid of certain other plant constituents, particularly calcium salts. We have sometimes further fractionated an impure sample of virus by precipitation with 50 % alcohol in the presence of added calcium chloride, but after such treatment it is often difficult to get the virus back into solution.

Coloured virus preparations that will not layer can, however, always be further purified by incubation with trypsin. Solutions containing about 0·5–2 % of solids and 0·2 % of commercial trypsin with a drop of chloroform added as a disinfectant have been incubated at 37° C. and at pH 8 for from 10 to 30 hr. This treatment has given invariably a product that becomes colourless after two to three further precipitations with acid and dilute ammonium sulphate solution. No differences have been noticed between material purified with trypsin and that prepared without, but the possibility that after treatment with trypsin the final product may be a complex cannot be excluded. The difference in size between the virus particle and the trypsin molecule is, however, so great that the combination of one molecule of the enzyme to one particle of the virus might well have little effect on the physical properties of the virus.

The yield of purified virus obtained has varied from 1 to 2 g/l. of expressed sap. The yield is greater from plants grown in the summer, and is greatest from young actively growing plants that have been infected for about 3 weeks to a month. From such plants also it is easier to isolate the virus, and all the steps described above are not necessary. The sap from young tobacco and tomato plants is not highly pigmented and the treatment with alcohol can be omitted, the virus being precipitated directly from the clarified sap with either acid or ammonium sulphate. The brown material (largely carbohydrate), which in the previous method is rendered insoluble by the alcohol, can now be removed by treatment with ammonium sulphate. Some is not precipitated with a quarter saturation, and some does not dissolve after precipitation. The ammonium sulphate precipitate, therefore, should be suspended in about two to three times its volume of water and centrifuged until only slightly opalescent in transmitted light. By reflected light the supernatant fluid should have an intense sheen. The virus can now be freed from the ammonium sulphate by repeated precipitation with acid.

In recent preparations we have found that a considerable saving can be effected by heating the crude infective sap to 70° C. before clarifying it. This produces a precipitate that aggregates quickly and can be removed by a few minutes' centrifuging or by filtration. This treatment not only clarifies the sap much more readily than the other methods but it also gets rid of considerably more of the plant constituents. After heating, the virus can readily be isolated from the supernatant or filtrate by direct precipitation with acid or ammonium sulphate, and the treatment with alcohol can safely be omitted. The yield resulting from this method is good, and when large quantities of sap are handled it is more convenient than the other methods.

ANALYSIS OF THE PURIFIED VIRUS PREPARATIONS

Dried neutral solutions of the three virus strains have analytical compositions falling within the following range:

	%		%
Carbon ...	49·3–50	Phosphorus ...	0·45–0·55
Hydrogen ...	7·2–7·4	Ash	1·5–3·0
Nitrogen ...	14·4–16·6	Carbohydrate ...	2·5
Sulphur ...	0·24–0·59		

Part of the ash is produced by the sodium hydroxide used for the neutralization, but even after prolonged dialysis of the virus preparations at about *p*H 3·3 some ash remains. One gram of these proteins requires from 0·2 to 0·23 millimols of NaOH to take it from *p*H 3·3 to 6·5, and their apparent equivalent weight is therefore about 4000. Phosphorus estimations were carried out by the Fiske and Subbarow method (1925) after incineration with sulphuric acid and perhydrol. This method was adopted after it was found that the Pregl method gave very unreliable results. The phosphorus content, however, has been confirmed by precipitation both as phosphomolybdate and as ammonium magnesium phosphate. The carbohydrate estimations were made by an orcin method (Pirie 1936) based on that of Tillmans and Philippi (1929). No consistent differences have been noticed between preparations of the three different virus strains.

When dried at *p*H 3·3 a preparation of Enation mosaic virus had the following composition: carbon 51·2 %, hydrogen 7·1 %, nitrogen 16·7 %, sulphur 0·26 %, phosphorus 0·51 % and ash 1 %. The figures for carbon, hydrogen and nitrogen fall within the range usual for proteins, and are similar to those published by Stanley (1936*a*) in his final paper on tobacco mosaic virus; then, however, he found neither phosphorus nor sulphur, and he makes no mention of carbohydrate. More recently (1937) he has agreed that his preparations of aucuba mosaic virus contain the amount of phosphorus and sulphur that we find, and it seems probable that further work will show that his tobacco mosaic virus preparations are very similar also. The sulphur contents of our preparations are variable. Although we have never had a preparation containing less than 0·2 % of sulphur it is possible that this may be an impurity, but it cannot be removed either by dialysis or by the prolonged centrifuging of a virus solution containing barium ions. Also, it follows the virus persistently through the various other purification processes that will be described.

The phosphorus and carbohydrate contents of our preparations are very constant. They are unaffected either by prolonged dialysis in cellophane tubes against dilute acid or alkali, or by reprecipitation ten times with either acid or quarter-saturated ammonium sulphate solution. From precipitates with their antisera, and with papain and clupein sulphate, the viruses have been recovered with their full activity and with their phosphorus content unaltered, and incubation with trypsin preparations rich in nuclease has no effect on the phosphorus content. All the treatments that we have tried which in no way inactivate the virus preparations leave the phosphorus content unaltered. Some treatments that do inactivate them, such as heating to 90° C. or exposure to strong acid or alkali, split off a nucleic acid or its breakdown products. Other treatments, however, that also inactivate them have no effect on the phosphorus content, e.g. nitrous acid, which destroys the infectivity without affecting the serological activity of the preparations, and drying, which affects both. We are therefore unable to agree with the statement of Stanley (1937) on aucuba mosaic virus, that the nucleic acid is merely a contaminant and that it is inessential to activity.

FURTHER PURIFICATION OF THE VIRUS PREPARATIONS

It has already been stated that solutions containing more than about 4% of the proteins, prepared as described above, after standing for some time will separate into two layers (fig. 1, Plate 12). The two layers have different solid contents and different appearances, both when viewed by ordinary light and through crossed Nicol prisms. The upper layer is the more dilute, and if separated from the lower and further concentrated, either by cautious evaporation in a vacuum or by precipitation with acid and resolution in a smaller volume of water, it will give more of the lower layer. Perhaps the simplest method of getting more lower layer is to freeze the upper-layer fluid in a centrifuge tube and then allow it to thaw while centrifuging.

The upper layer is slightly opalescent by transmitted light, and behaves like a suspension of anisotropic rods or plates that can be orientated by streaming. The stationary upper-layer fluid merely rotates the plane of polarization of polarized light, but fluid in which the particles have been orientated changes plane-polarized light into elliptically polarized light; solutions of myosin give a similar but weaker effect (Von Muralt and Edsall 1930). Those regions in the upper layer where there is a shear, and in which the particles are therefore orientated, become birefringent and are visible between crossed Nicol prisms; this is illustrated in fig. 2, Plate 12.

The lower layer is spontaneously birefringent (fig. 1, Plate 12); it may be perfectly clear by transmitted light, but it has a strong sheen by reflected light. The lower-layer fluid when separated from the upper can be mixed with a considerable volume of water and still be liquid crystalline if examined immediately in polarized light. A few minutes after diluting, however, it begins to change, and after standing for some time once more separates into two layers. The lower layer is again liquid crystalline, but now has a smaller solid content. Because of the small specific gravity differences involved the droplets of each layer move very slowly into the other, and it may take many hours for the interface between the two layers to become quite sharp (figs. 3 and 4, Plate 13). When the process of separation has started it can be accelerated by centrifuging, but the initial conversion of the unstable fluid into two layers proceeds faster if the solution is allowed to remain quite still. The actual separation into layers also proceeds faster at 37° than at 0° C.

The process of diluting a bottom-layer fluid and separating off the new, more dilute bottom layer can be repeated until the concentration of solid in the bottom layer is reduced to about 1·6 % with tobacco mosaic virus and Enation mosaic virus. With aucuba mosaic virus the limiting dilution is somewhat less, and the critical concentration is about 2·2 %. These values probably have no absolute significance, and improvements in the methods of purification may lead to even lower figures. The critical concentration at which a solution becomes spontaneously birefringent is dependent to some extent on the temperature, and dilute solutions that are liquid crystalline at 0° C. may lose this property at room temperature and regain it when cooled.

In addition to being the more concentrated, bottom-layer material also seems to be the more pure, and the processes of dilution of bottom layer, which lead to a decrease in the concentration at which a virus solution will become spontaneously birefringent, usually also lead to an increase in the serological titre of the preparation. In general, however, there is no corresponding increase in the infectivity, suggesting that the units in the lower layer may possibly be larger aggregates than those in the upper, and there is no appreciable change in the analytical figures. Table II shows the results of three comparisons of the serological activity and the infectivity of top and bottom layers which separated from virus solutions prepared by precipitation with acid and ammonium sulphate.

TABLE II—ACTIVITY OF "TOP" AND "BOTTOM" LAYERS IN EQUILIBRIUM

			Infectivity at various dilutions Average no. of lesions per half-leaf			
Virus	Layer	Serological titre	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Tobacco mosaic	Top	$1/6 \times 10^6$	165	99	23	4
	Bottom	$1/10^7$	161	80	25	2·6
Aucuba mosaic	Top	$1/6 \times 10^6$	144	51	24	5
	Bottom	$1/10^7$	151	74	26	4
Enation mosaic	Top	$1/3 \times 10^6$	75	27	6·5	0·16
	Bottom	$1/6 \times 10^6$	72	27	7·5	0·5

Dilution given as weight of protein in grams per c.c.

Towards the end of the process of diluting successive bottom layers the difference in concentration between the two layers in equilibrium becomes smaller and smaller, and with tobacco mosaic and Enation mosaic viruses may be only one part in a hundred when the concentrations are about

1·8 %. With more concentrated preparations the difference is much larger, and a lower layer containing 8 % of solids may be in equilibrium with an upper layer containing only 4 %. The precise values depend on the amount of impurities present.

From these dilution experiments it is apparent that solutions of tobacco mosaic and Enation mosaic virus purified by the use of strong salt solutions and acid can exist in the liquid crystalline state if they have a solid content greater than 1·6 %, and that with aucuba mosaic virus a slightly higher concentration is necessary. If solutions of the purified viruses are not spontaneously birefringent when the solid content is from 2 to 3 % they can be still further purified, if purity be estimated on the basis of serological activity. All virus preparations which give dilute liquid crystalline solutions, however, do not necessarily give uniformly high precipitation end-points. Some old preparations of tobacco mosaic virus with serological titres of only $1/4 \times 10^6$ became slightly gelatinous and gave spontaneously birefringent solutions with a solid content of but 1 %, and the addition of certain substances, notably glycerophosphates and hexosephosphates, to fresh virus preparations has a similar effect. These phenomena, however, have not been investigated in detail.

The further purification of solutions of these viruses which, although relatively concentrated, are not liquid crystalline cannot be effected by precipitations with either acid or ammonium sulphate, for the contaminants (presumably breakdown products of the virus) precipitate under the same conditions and in the same apparently crystalline manner as the virus. For further purification it is necessary either to dilute successive fractions of bottom layer as described above, to incubate with trypsin, or to separate a product by centrifuging at high speed. The last method will now be discussed in more detail.

BEHAVIOUR OF THE VIRUS PREPARATIONS IN A CENTRIFUGAL FIELD

The general appearance of solutions of the three virus strains is the same whether they are dissolved in water or in dilute salt solutions, and the viruses cannot be removed from such neutral solutions by centrifuging at 3500 r.p.m. When either the salt concentration or the acidity is increased the fluids become opaque, and at a salt concentration that depends somewhat on the purity of the virus preparation and on the salt used it becomes possible to sediment the virus at this speed. Virus solutions to which insufficient acid for complete precipitation has been added have a different appearance from those to which insufficient salt has been added.

With acid the fluid becomes opaque and slightly rigid, but it shows no very noticeable sheen; this slightly gelatinous fluid is thixotropic. With a little ammonium sulphate the fluid becomes somewhat gelatinous and develops a very intense sheen, the sheen being obvious even when the fluid is at rest. Virus preparations sufficiently purified to give a birefringent layer at a concentration of 4% will give a precipitate which can be centrifuged out at 3500 r.p.m. when they are only one-eighth saturated with ammonium sulphate. By increasing the concentration of ammonium sulphate to one-sixth or one-fifth saturation the precipitate can be sedimented much more rapidly.

The optimal precipitation point with acid was found by centrifuging virus solutions at various *p*H values, and testing the supernatant fluids for their infectivity and serological activity. In these tests no indication was obtained that virus activity could be separated from the protein, and the infectivity and serological activity of the supernatant fluids were found to be inversely proportional to the amount of precipitate obtained. The optimal precipitation point was found to depend on the salt content of the virus solutions. In the presence of a little salt, tobacco mosaic virus precipitates completely at *p*H 3·4, but it can be precipitated over a considerable range on either side of this point by intense centrifuging. This is in good agreement with the *p*H value given by Stanley (1936*a*) for the isoelectric point of tobacco mosaic virus, and with the results of Best (1936) and Eriksson-Quensel and Svedberg (1936). In the absence of salts, or with a salt concentration of less than M/50, however, the apparent isoelectric point moves to about *p*H 4·2, but becomes even less precise than it is in the presence of salt. As previously stated the salt-free precipitate at *p*H 4·2 differs in appearance from that obtained in the presence of salt at *p*H 3·4, and it is apparently amorphous. No differences large enough to be of value for distinguishing between the three virus strains have been detected.

Bechold and Schlesinger (1933) showed that it is possible to sediment tobacco mosaic virus from clarified infective sap and, assuming a specific gravity of 1·12, they calculated the diameter of the particle as 500 A. Using Stanley's purified virus preparations Eriksson-Quensel and Svedberg (1936) have found a similar value. Wyckoff and Corey (1936) have confirmed the fact that it is possible to centrifuge the virus out of clarified sap, and they have pointed out that the precipitate is birefringent. We have described briefly (Bawden, Pirie, Bernal and Fankuchen 1936) the separation of a jelly-like layer by centrifuging our purified virus solutions. In the experiments to be described below an Ecco-Blitz angle centrifuge, giving a centrifugal field of 16,000 times gravity at the bottom of the tubes turning at a speed of 14,000 r.p.m., was used; it is therefore difficult to calculate values for the sedimentation constant from the data obtained. Furthermore, the viscosity and degree of mixing of the fluids centrifuged are uncontrolled factors. The optical properties of purified virus solutions suggest that the particles are rod-shaped, and it would, therefore, be impossible, at the present time, to deduce the particle size from the sedimentation constant even if the latter were measured accurately. Valuable centrifugal data can perhaps be obtained from untreated clarified infective sap if it should prove that the virus particles occur there in a more or less spherical state, but it has not seemed worth while to give any great precision to measurements on the purified and probably aggregated material, nor to look for minor differences in the sedimentation rates of the three virus strains.

When clarified infective sap is centrifuged at this speed a birefringent jelly-like layer is deposited. The rate of sedimentation, however, is slow, and as the volume of sap that can be centrifuged in this manner is small it is not at the present time a practicable method for the isolation of the virus.

Virus solutions in the two states which we have called top layer and bottom layer respectively behave differently when centrifuged at 14,000 r.p.m. Top-layer solutions deposit simply a viscous layer of jelly, the solid content of which depends on the concentration of the solution put into the centrifuge tube. By centrifuging for from 1 to 3 hr. it is possible to sediment most of the virus from a neutral solution, and to leave a more dilute solution of virus covered by a layer of water from 0·5 to 2 cm. deep. Table III shows the positive correlation between the solid content of the jelly and that of the fluid from which it was sedimented. Within the limits mentioned, the time of centrifuging does not affect appreciably the concentration of the jelly layer but only the total amount deposited. This is shown in Table IV. There is, of course, a small secondary effect produced by changes in the concentration of the fluid during the period of centrifugation.

Evidence that the particles in these purified virus preparations are built up by the linear aggregation of smaller particles will be presented later. Attempts to get centrifugal evidence for any variability in the length of the particles have so far failed. If the supernatant fluids from one centrifugation are poured off and then centrifuged again, they behave in exactly the same manner as did the original fluid at the same concentration. There is, therefore, no gross inhomogeneity in our preparations, but this method of testing is not sufficiently sensitive to detect an inhomogeneity of the type reported by Eriksson-Quensel and Svedberg (1936). Similarly, if the supernatant fluid be divided into two, and one-half precipitated with ammonium sulphate and acid and then redissolved in its original volume of fluid, no differences can be detected between the centrifugal behaviour of the reprecipitated and the original solution by this method.

TABLE III—EFFECT OF THE CONCENTRATION OF THE CENTRIFUGED FLUID ON THE SOLID CONTENT OF THE JELLY DEPOSITED

Initial concentration of centrifuged fluid	Wet weight of sediment mg.	Dry weight of sediment mg.	% solid in sediment	% of total virus sedimented
		A—Tobacco mosaic virus		
6·2	191	45	23·5	25
3·7	171	35	20·5	32
2·25	161	27	17	40
1·35	140	21	15	52
1·02	118	15	13	49
0·71	107	12	11	56
		B—Enation mosaic virus		
4·0	128	36	28	30
2·6	119	31	26	40
1·0	77	17	22	57
0·7	52	10	19	48
0·53	41	7	17	44
0·41	35	5	14	41

Each tube contained 3 c.c. of fluid at *p*H 7, and was centrifuged for 2 hr. at 14,000 r.p.m. and at 23·3° C. Although concentrated, none of these fluids was spontaneously birefringent, i.e. they were all in the state we have called top layer.

TABLE IV—EFFECT OF DURATION OF CENTRIFUGATION AT 14,000 R.P.M. ON THE SEDIMENT

Time min.	Wet weight of sediment mg.	Dry weight of sediment mg.	% solid in sediment	% of total virus sedimented
		A—2·5 % solution of tobacco mosaic virus		
192	160	40	25	53
127	96·5	24	25	32
58	39	10	25	13
23	12	3	25	4
		B—1·3 % solution of Enation mosaic virus		
180	122	31·4	26	80
130	100	25	25	64
100	81	19	23	49
50	35	9	26	23

Each tube contained 3 c.c. of fluid, in the top-layer condition, at *p*H 7.

Table V shows the effect of centrifuging virus solutions of the same concentration at a number of different *p*H values. Below *p*H 5·6 the solutions become gelatinous, this being the first stage in the formation of the acid precipitate previously described.

TABLE V—EFFECT OF *p*H ON THE AMOUNT OF SEDIMENT

Buffer	Wet weight of sediment mg.	Dry weight of sediment mg.	% solid in sediment	% of total virus sedimented
*p*H 5·6 phthalate	179	52	29	75
*p*H 6·1 phthalate	128	37	29	54
*p*H 6·6 phosphate	115	33	29	48
*p*H 7·1 phosphate	104	32	31	46
*p*H 7·6 phosphate	96	30	31	43
*p*H 9·1 borate	93	28	30	41

Each tube contained 3 c.c. of top-layer tobacco mosaic virus 2·3 % and was centrifuged for 2 hr. at 14,000 r.p.m. in M/15 buffer at 23 ± 3° C.

The spontaneously birefringent virus solutions which we have referred to as bottom layer centrifuge in a rather different manner. The jelly deposited may have less than half the solid content of that sedimented from a top-layer solution of the same concentration. There is no clearly defined interface between the jelly and the concentrated bottom layer that lies immediately above it; there is likewise no sharp interface between the bottom layer and the top layer which forms during centrifuging, but merely a diffuse region in which particles of the one layer are found dispersed in the other. A division, however, can be distinguished where the system changes from a suspension of top-layer material in bottom-layer material to a suspension of bottom-layer material in top-layer material.

The relationship that has been shown between the solid content of the jelly and that of the centrifuged fluid is interesting, for by varying the concentration of the centrifuged fluid it is possible to get stable jellies with very similar physical appearances, but with solid contents varying between 10 and 35 %. A rather limp jelly with a solid content of only 7 % has sometimes been precipitated. These jellies are highly birefringent, and are completely orientated when they are sucked into capillary tubes of about 1 mm. diameter. The results of X-ray examinations of these jellies and the nature of the forces which hold the particles apart as an oriented jelly in spite of the intense gravitational fields will be discussed later.

High-speed centrifugation can be used as a method of further fractionating these virus preparations. Preparations that have been partially inactivated by drying cannot be fractionated by this treatment, but those made by the precipitation methods described, which will not give a birefringent layer unless more concentrated than 5 %, will always deposit on high-speed centrifugation a jelly which dissolves to give birefringent solutions at about 2 %. By this method of fractionation more than a half of the total virus can readily be recovered in a state in which it gives dilute liquid crystalline solutions, whereas if the further purification is carried out by the method of repeatedly separating and diluting successive bottom layers only a small part of the virus is recoverable in the final bottom layer. When, however, the risk of a possible contamination with trypsin is of no importance, incubation with trypsin is by far the most convenient method of obtaining the virus in the highly birefringent state.

Dilute solutions of the jellies obtained by high-speed centrifugation of top-layer material, on standing, again separate into two layers. Again, the lower layer is spontaneously birefringent while the upper is not. The serological activity and infectivity of top- and bottom-layer material obtained in this manner have been found to be the same. This fact is readily explicable if the jelly sedimented from partially purified preparations is homogeneous, i.e. if the high-speed centrifugation effects a further fractionation similar to that produced by dilution of successive bottom layers. The results of two tests on such layers are given in Table VI.

TABLE VI—COMPARISON OF THE SEROLOGICAL TITRE AND INFECTIVITY OF TOP AND BOTTOM LAYERS FROM DILUTE SOLUTIONS OF THE JELLIES OBTAINED BY CENTRIFUGING TOP-LAYER MATERIAL AT 14,000 R.P.M.

			Infectivity on *Nicotiana glutinosa* Average no. of lesions per half-leaf			
Virus	Layer	Serological titre	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Tobacco mosaic	Top	$1/8 \times 10^6$	134	66	25	2·2
	Bottom	$1/8 \times 10^6$	129	72	23	2·6
Enation mosaic	Top	$1/6 \times 10^6$	122	45	8	2
	Bottom	$1/6 \times 10^6$	114	46	7	3

ABSORPTION SPECTRA

The ultra-violet absorption spectra of the three virus strains have been measured using a Spekker ultra-violet spectrometer and 2 cm. column of 0·02 % virus solution. No definite differences have been observed between the spectra given by the different strains, and the spectra do not differ appreciably from those usually obtained with proteins. Fig. 5 is the absorption curve of a solution of aucuba mosaic virus.

SPECIFIC GRAVITY

The specific gravity of tobacco mosaic virus has been measured in three different ways. First, particles of the dried virus have been suspended in mixtures of nitrobenzene and dichlorbenzene, and the ratio of the two adjusted until the particles were in equilibrium. Secondly, the precipitate obtained by adding ammonium sulphate to virus solutions has been equilibrated in mixtures of sucrose and ammonium sulphate solution (Adair and Adair 1936). Thirdly, the specific volume of the virus has been determined from the specific gravity of a dilute solution of known concentration. The first two methods gave the specific gravity as 1·29–1·31, and this is in good agreement with the figure usually obtained with proteins.

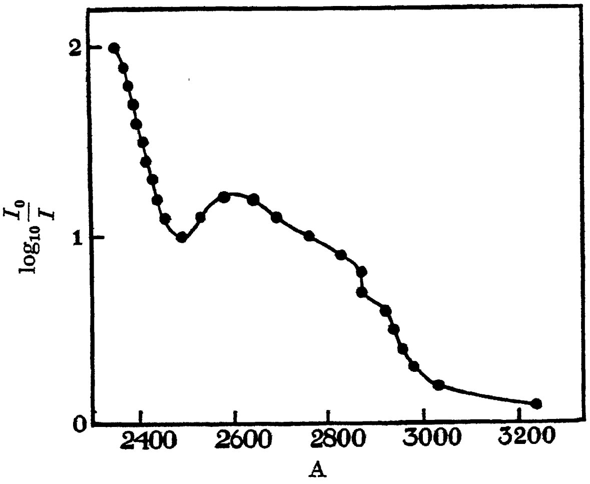

FIG. 5

Eriksson-Quensel and Svedberg (1936) have stated that the specific volume of tobacco mosaic virus protein is 0·646, i.e. the specific gràvity is 1·55, and they comment on the fact that this is an unusual value for a protein. Our chemical observations on the virus preparations have shown no abnormality that might account for such a high specific gravity, and our measurements at 20° C. have given a specific volume of 0·73. This figure corresponds to a specific gravity of 1·37, and agrees well with the values obtained by this method with other proteins (Chick and Martin 1913). The differences that we have found between the specific gravity values as determined by the different methods also agree well with their observations. All three virus strains have given the same values for specific gravity.

Table VII shows the results of a few determinations of the specific volume of the three strains. These were measured by adding water to a 3–4 % solution of neutral salt-free virus until a glass float was in equilibrium at the temperature stated. The specific gravity of the float at that temperature was then measured by equilibrating it in solutions of sodium chloride of known concentration, and the values given in Landolt and Börnstein's tables for the specific gravity of sodium chloride solutions were used. From 1 to 2 c.c. of the virus solution was then frozen, dried and weighed to obtain its exact concentration. The value given in Table VII for a 26 % solution of tobacco mosaic virus was derived from a direct observation of the weight of a known volume of virus jelly.

TABLE VII—THE SPECIFIC VOLUMES OF THE VIRUSES

Strain	Concentration in g./100 ml. of solution	State	Temp. ° C.	Specific gravity of virus solution	Specific volume of virus
Tobacco mosaic	1·73	Top	20	1·0029	0·73
	1·88	Top	20	1·0033	0·73
	26·0	Jelly	15	1·07	0·73
Enation mosaic	1·61	Top	18	1·0025	0·76
	1·75	Top	19	1·0032	0·73
	2·49	Top	12·5	1·0065	0·72
	2·85	Bottom	18·5	1·0063	0·73
Aucuba mosaic	1·61	Top	16	1·0030	0·75
	2·78	Top	15	1·0064	0·74
	2·95	Bottom	20	1·0062	0·73

GELS, FIBRES AND "CRYSTALS" OF THE VIRUS

The satin-like sheen produced in solutions of these purified viruses when they are acidified or mixed with a quarter of a volume of saturated ammonium sulphate solution suggests that the material precipitated may be in the crystalline state. The individual particles of the precipitate, however, are too small to permit a conclusive microscopical examination. Attempts have been made, therefore, to increase the size of the "crystals" by slow precipitations; these attempts failed, but in the course of the work it became increasingly obvious that the first process in the production of the "crystals" is the formation of a gel, and that this breaks up when stirred or shaken and in its place are formed the small "crystals" or fibres with the characteristic sheen.

Phthalate buffer at *p*H 3·3 has been diffused into neutral virus solutions, acetate buffer at pH 4 has been diffused into acid virus solutions and half-saturated ammonium sulphate solution has been diffused into neutral virus solutions. In each experiment a skin of jelly first precipitated at the interface, and then the remainder of the virus solution gradually turned to a limp jelly with no crystalline appearance until it was shaken or stirred.

When one volume of saturated ammonium sulphate solution is added to six or seven volumes of a dilute virus solution no precipitate is produced, but the fluid develops a very intense sheen and becomes slightly thixotropic. If this fluid is now allowed to evaporate slowly over calcium chloride it does not alter appreciably in appearance, but it is soon converted into a rigid jelly. This jelly when broken up by stirring changes into the usual "crystalline" precipitate got with ammonium sulphate.

The process of gel formation can be more easily studied in mixtures containing 0·5–1·0% of the virus, 0·25–0·5% of glycine and 5% of neutralized ethyl formate. The pH is adjusted initially to about 5, and the fluid is immediately put into the vessels in which it is to be observed. After a time, depending on the initial pH value, the glycine concentration and the temperature, sufficient ethyl formate will have undergone hydrolysis to give a mixture at about pH 3·3. During the acidification the optical properties of dilute virus solutions which have been kept quite still undergo no change, although the mixture turns to a fairly rigid jelly. When shaken or stirred this jelly breaks up and changes almost instantly to a suspension with the usual sheen. If now allowed to remain quite still the mixture does not return to the gel state, but it behaves exactly like the precipitate that is produced by adding acid directly to virus solutions, and it has the same appearance under the microscope. When more concentrated virus solutions are slowly acidified in this way they behave somewhat differently, for the undisturbed gel breaks up into a mosaic of irregularly disposed birefringent spindles. When stirred, however, this again gives rise to a suspension with the characteristic sheen.

These phenomena suggest that it is more accurate to describe the visible virus precipitates that are obtained with acid and ammonium sulphate as fibrous rather than as crystalline. The regular appearance of the fibres under the microscope could then be attributed to the more or less regular breaking up of a gel rather than to the wholly regular process of crystal formation (cf. Bernal and Fankuchen 1937).

From most points of view this distinction is not perhaps an important one, but if it be accepted it prevents us from adducing the apparent crystallinity of these virus preparations as evidence for their purity. Certain treatments can destroy infectivity without greatly affecting the physical properties of these viruses. Such preparations give both ammonium sulphate precipitates and liquid crystalline solutions similar in appearance to those given by fully active virus. Other treatments, such as heating or exposure to alkali, which inactivate and dephosphorylate the protein, lead to a loss of the ability to give these crystalline appearances. Preparations partially inactivated by these methods, and therefore having a rather low activity and phosphorus content, may give with ammonium sulphate an appearance of crystallization very similar to that seen in fully active virus preparations. These contaminated preparations cannot be purified by further precipitations (or "recrystallizations") with ammonium sulphate, but they can be further purified by the other methods we have described.

THE EFFECT OF HEAT ON THE VIRUS PREPARATIONS

The coagulation of crude infective sap when heated to 70° C. has already been described. When dilute solutions of the purified virus preparations, protected from evaporation, are heated at 70° C. for as long as 30 min. there is no perceptible change in their appearance or in their ability to give anisotropy of flow. When tested at 60–70° C. dilute solutions give rather less anisotropy of flow than when examined at room temperature, but when cooled they are found to be unchanged. Similarly, if more concentrated solutions consisting of mixtures of top and bottom layer are heated to 70° C. the ratio of the two layers is unaltered.

When neutral solutions are heated to higher temperatures (75–80° C.) a fairly rapid change sets in; the fluid becomes opaque and soon turns to a gel, the rigidity of which naturally depends on the concentration of the virus solution heated. Two minutes at 80° C. will turn a 2% spontaneously birefringent solution to one showing only anisotropy of flow, and 4 min. will carry the decomposition far enough to render the fluid opaque. No differences have been noticed between the thermal labilities of the three virus strains.

Heating for a few minutes at 90–95° C. causes a coagulation and a complete loss of the ability to give anisotropy of flow: this treatment also destroys the infectivity and serological activity of the virus preparations. When a neutral solution in the presence of a trace of salt is heated for 5 min. at 95° C. the *p*H shifts slightly to the acid side, and the major part of the protein precipitates as a coagulum that can be centrifuged off. This precipitate is free from both phosphorus and carbohydrate. The supernatant fluid still contains some protein, part of which can be precipitated by the addition of from 0·5 to 1·0% of a neutral salt such as sodium chloride or ammonium sulphate. The supernatant fluid now contains very little protein but all the phosphorus and carbohydrate content of the virus preparations. When mineral acid is added to the supernatant a curdy precipitate separates immediately, and soon aggregates into resinous masses with the characteristic appearance of nucleic acid. When denatured by heating (or by the other agents such as glacial acetic acid and pyridine, the effects of which will be described later) protein and nucleic acid are the only breakdown products that have been found. If there are any other constituents of these virus preparations they must be relatively minor ones. The properties of these two substances will now be described only superficially, for their more detailed examination must await the preparation of much larger quantities of the virus.

The proportions in which the soluble and insoluble proteins are found after denaturation by heating vary somewhat with the conditions of heating and with the *p*H value of the solution. The soluble protein can be removed by precipitation at *p*H 4·5, but if removed in this manner most of the nucleic acid is lost. When hydrolysed by acid these proteins behave like most other proteins, and the amino-nitrogen content of the hydrolysate reaches a maximum after 20 hr. boiling with 4N HCl. They give all the protein colour reactions, and have an arginine content (as estimated by Weber's (1930) modification of the Sakaguchi reaction) of from 6 to 7 %. This reaction was used to follow the distribution of the protein in most of the experiments on the precipitation or enzymic hydrolysis of the protein. Negligible amounts of material were extracted from these proteins by prolonged extraction in a Soxhlet with ether, acetone, alcohol or pyridine. The proteins obtained from heat-denatured preparations of the different virus strains have shown no appreciable differences from each other.

The resinous masses of nucleic acid are readily converted to a loose powder by grinding with water. Neutral solutions of the sodium salt are convenient to handle, and they give a solid with a light open texture if dried while frozen. This material does not react with the virus antisera, nor does it in any way affect the precipitation of virus and antiserum.

Prepared in this way the nucleic acid product contains from 4 to 7 % of phosphorus and from 24 to 30 % of carbohydrate as estimated by the orcin method. Without previous hydrolysis it reduces potassium ferricyanide, under the conditions of the Hagedorn-Jensen reducing sugar estimation, to an extent corresponding to a reducing sugar content of 5 %. It also gives biuret and Sakaguchi reactions corresponding to from 10 to 20 % contamination with virus protein. This protein, judging from the Sakaguchi reaction, can be removed almost completely by prolonged centrifugation at pH 3, or, less conveniently, by careful treatment with dialysed iron.

The purified virus nucleic acid resembles yeast nucleic acid closely; it contains a pentose and does not give the reactions with Schiff's reagent characteristic of a desoxy pentose. The phosphorus is liberated as phosphate on acid hydrolysis in two stages in the manner described by Jones (1920) for yeast nucleic acid. The question of the relationship between these virus nucleic acids and yeast nucleic acid will be dealt with in a later paper, but it may be said now that the molecule is larger than that of yeast nucleic acid prepared in the usual ways, for it is retained on collodion membranes which readily permit the passage of yeast nucleic acid. It is possible that this difference is simply the result of the more extensive degradation suffered by yeast nucleic acids during the course of isolation, for the methods used for the isolation of virus nucleic acid are much gentler than those necessary for the isolation of yeast nucleic acid.

EFFECT OF DRYING

It is well known that leaves of plants infected with strains of tobacco mosaic virus can be dried and still be infectious. Our preparations of the three strains are also active after drying in air or over phosphorus pentoxide. The activity, however, whether measured by the serological or by the infection method, is reduced to one-half or one-third by one such drying; by redissolving and then drying again the activity may be still further reduced. The loss in activity following on one drying over P_2O_5 is shown in Table I. After seven dryings in one experiment with tobacco mosaic virus the activity fell to approximately 1/100 of that of the original undried material. The loss of activity is the same whether the solutions are dried at neutrality or at the acid precipitation point. It is immaterial whether the solutions are dried unfrozen, or after being frozen slowly, or after being frozen rapidly in liquid air. Material dried frozen is, however, in the most convenient state for further handling, and we always use this method in getting dry weights. Frozen material which has been dried *in vacuo* over P_2O_5 for a day at room temperature loses less than 0·3 % of its weight on subsequent drying at 100° C. Repeated freezing and thawing has no apparent effect on the activity of these viruses.

Drying over P_2O_5 destroys to a large extent the ability of the virus to give liquid crystalline solutions, and also greatly reduces the readiness with which solutions will show the phenomenon of anisotropy of flow. For example, a solution, which was liquid crystalline at 1·8 %, when dried once over P_2O_5 gave a solid which dissolved to give a solution containing only a trace of liquid crystalline material at 5·4 %, and none at 4·0 %. Virus that has been twice dried over P_2O_5 has never been observed to give a liquid crystalline solution at any concentration.

Solutions of dried virus resemble impure preparations of virus in many ways, and the properties of some unsuccessful preparations which are not highly active or which fail to give liquid crystalline solutions suggest that they are contaminated by virus breakdown products similar to those produced by drying the virus. The virus inactivated by drying precipitates in a manner similar to the active virus, and differs in this respect from the denatured product got by heating. Protein cannot be precipitated from a neutral solution of dried virus by the addition of a little salt, nor can nucleic acid be precipitated by acid. That part of the preparation which was inactivated by drying can be hydrolysed by commercial trypsin preparations, though much more slowly than can virus which has been denatured by heating. After digestion the part of the virus not inactivated can be isolated by precipitation with acid and ammonium sulphate. Without previous incubation with enzymes it has proved impossible to obtain virus from dried preparations giving either a high serological titre or capable of giving dilute liquid crystalline solutions either by precipitation with acid and ammonium sulphate or by centrifuging at 14,000 r.p.m. At this speed the dried material sediments in a similar manner and at about the same rate as the undried virus, but the precipitates obtained dissolve to give solutions which show anisotropy of flow only, and which are not liquid crystalline until more concentrated than 4–5 %.

This reproducible partial inactivation on drying is of interest because of the X-ray measurements on films of dried and partially dried virus. The course of the inactivation has therefore been followed in some detail. Samples of the same batch of tobacco mosaic virus were dried at atmospheric pressure over $CaCl_2$, $MgSO_4$ and $MgSO_4.7H_2O$, Na_2SO_4 and $Na_2SO_4.10H_2O$, and saturated ammonium sulphate solution. When redissolved the first solution was found to have lost half of its activity, the second one-quarter and the last two were unaffected. At 15° C. the mixture of $MgSO_4$ and $MgSO_4.7H_2O$ establishes a water-vapour pressure of 5 mm. and the Na_2SO_4 mixture a pressure of 9·2 mm. (figures from Landolt and Börnstein's Tables). Similarly, there is no loss of activity or crystallinity on drying, or even on repeated drying, over the Na_2SO_4, $Na_2SO_4.10H_2O$ mixture in the vacuum attained by a water-pump. After drying over sodium sulphate the virus retains 15–20 % of water, this can be removed by subsequent drying over phosphorus pentoxide. Drying in two stages in this way causes much less inactivation, whether measured by the capacity to give "bottom layer" or serologically, than direct drying over phosphorus pentoxide.

This curious behaviour can perhaps be explained in terms of the triangular shape deduced for the virus particles from X-ray measurements (Bawden, Pirie, Bernal and Fankuchen 1936), which indicated that the particles could pack together in two ways. It is possible that there is less mechanical destruction of the virus when the packing, which must take place during evaporation, is done gently.

The various methods of inactivating the virus seem to give rise to recognizably different products. By heating or treatment with acetic acid or pyridine, we get a nucleic acid and an insoluble denatured protein; when dried, a soluble nucleoprotein and no free nucleic acid; and when inactivated with acids or alkalis we get metaproteins and free nucleic acid.

The solid precipitated from fully active virus preparations at *p*H 3·4 in the presence of a little salt contains about 26 % of dry matter. In the absence of salts, even after long centrifuging at 3500 r.p.m. at *p*H values around the precipitation point, the precipitate cannot be packed so tightly. If, however, this loose sludge of virus is now drained without evaporation by centrifuging on a porous plate, the solid content can be raised to about 35 %. A jelly which also may have a solid content as high as 35 % can be deposited from neutral solutions of virus by centrifuging at 14,000 r.p.m. These treatments in no way affect either the activity of the virus or its capacity to produce dilute liquid crystalline solutions, and these are the greatest concentrations of virus we have obtained by mechanical means, i.e. without evaporation.

[*Note added in proof* 19 *June* 1937.—The acid precipitate can be converted into a translucent buttery mass with a water content of only 50 % by centrifuging for ½ hr. at 14,000 r.p.m. in the end of an L2 Chamberland filter candle. When soaked in water this mass regains its "crystalline" appearance.]

THE EFFECT OF ENZYMES

No enzyme preparation has yet been found that attacks these purified virus preparations at an appreciable rate, or that has any permanent effect on their infectivity, and we have found incubation with trypsin to be a most effective method of further purifying our products. We have incubated the virus preparations with commercial trypsin, "pancreatin", pepsin, papain and autolysed preparations of kidney at a number of *p*H values around the optima for enzymic activity. All these enzymes were shown to be strongly proteolytically active by testing them against heat-denatured virus which they rapidly hydrolysed, but they had no appreciable enzymic effect on the preparations of active virus. The behaviour of these strains of tobacco mosaic virus is, therefore, very different from that of potato virus "*X*" (Bawden and Pirie 1936), and our results are substantially the same as those obtained by Stanley (1934*a* and *b*) with crude infective sap. In the presence of large amounts of trypsin the infectivity of the purified virus preparations is almost destroyed while the serological activity is unaffected. This loss of infectivity occurs immediately the virus and enzyme are mixed, and no further loss follows incubation. By precipitation with acid or dilute ammonium sulphate solution, however, the virus can be recovered with its full activity from such non-infective mixtures. The material recovered from such mixtures seems to be identical with virus that has not been incubated with trypsin, but, as we have previously stated, the possibility that it may be a virus-trypsin complex cannot be definitely excluded.

The Recovery of Virus from Precipitates with their Antisera

When purified virus preparations are mixed with their antisera at optimal combining proportions a flocculent precipitate is produced, and the mixture is almost non-infective. A slight recovery of the infectivity can be made merely by diluting these mixtures. Chester (1936) has shown that the infectivity of crude infective sap and antiserum can be much increased by incubating them with pepsin.

If our purified virus preparations consist of a mixture of active virus and inert protein, or of a protein and a contaminating nucleic acid, it is to be expected that the precipitation with specific antisera might perhaps alter the ratio of the components. We have found no evidence for this, and material recovered from precipitates with antiserum after digestion with pepsin is apparently the same as our normal virus preparations.

The precipitate obtained by incubating 32 mg. of purified tobacco mosaic virus with 3 c.c. of antiserum was centrifuged off, suspended in water and heated for 5 min. at 78° C. The precipitate did not appear to coagulate, and after this treatment trypsin had little or no effect on it. After incubation at 37° C. at about *p*H 3 with pepsin, however, the fluid developed an intense anisotropy of flow, and after a few hours' incubation, material with the characteristic appearance of the purified virus preparations could be centrifuged off. 15 mg. of this material were recovered; it contained 0·5 %

phosphorus and 2·5 % carbohydrate, gave a serological titre of $1/4 \times 10^6$ and was infective at a dilution of 10^{-8}.

In a similar experiment using 50 mg. of a purified Enation mosaic virus preparation a final yield of 37 mg. was isolated. This showed good anisotropy of flow, had a phosphorus content of 0·48 %, gave a serological titre of $1/2 \times 10^6$ and was also infective at a dilution of 10^{-8}.

THE EFFECT OF VARIOUS SUBSTANCES ON THE VIRUS PREPARATIONS

Stanley (1935*b*) has described the effect of 110 substances on the infectivity of partially purified tobacco mosaic virus, and he has reviewed the literature on the subject in some detail. It appears from this that the agents which inactivate the virus are, in general, strong acids and alkalies, oxidizing agents and protein precipitants. In this section the effects of a miscellaneous group of substances on the purified virus preparations will be described.

Solvents

We have already shown that the virus is precipitated from plant sap by the addition of an equal volume of alcohol. When, however, salt-free solutions of the purified virus at *p*H 7 are mixed with alcohol there is no precipitate until the alcohol concentration is greater than 80 %. Denaturation proceeds slowly at this alcohol concentration. Concentrated solutions containing 30 % of purified virus can be dissolved in 80 % alcohol as easily as in water. The presence of salt, however, alters the behaviour greatly, and from certain salt solutions the virus can often be precipitated completely with 40 % alcohol. Calcium ions are more effective in producing this precipitate than the others that we have tried. Acetone has an effect similar to that of alcohol, but it denatures the virus more readily.

When dilute acetic acid is added to solutions of the three virus strains the characteristic acid precipitate already described is produced. When, however, solutions of virus are added to four or five parts of glacial acetic acid an irreversible change occurs, and there separates a precipitate of nucleic acid, apparently identical with that obtained by heating the virus preparations to 90° C. The clear supernatant fluid deposits on evaporation a phosphorus-free protein as a perfectly transparent, isotropic, tough film. This wets with difficulty and is only slightly soluble in water even when neutralized. The solution obtained does not react with the virus antiserum.

In small quantities pyridine has no effect on the virus preparations, but when the concentration is raised to 25–30 % a rapid denaturation sets in.

Initially such mixtures show the usual faint opalescence of the purified virus solutions, and they give anisotropy of flow, but after a few minutes they go quite clear and lose their ability to give anisotropy of flow. The addition of a little ammonium sulphate solution to this clear fluid causes the precipitation of denatured protein that is phosphorus-free and is soluble in water only in the complete absence of salts. From the supernatant fluid, nucleic acid can be precipitated by the addition of acid.

The instability of these viruses in the presence of solvents makes it difficult to assess the value of some of the analytical figures which have been published. The analyses have often been carried out on acetone-treated preparations, and no mention is made of the activity after this treatment. Such analyses may approximate more closely to analyses on phosphorus-free denatured protein rather than to analyses on the virus.

Urea

It is well known that concentrated solutions of urea readily denature proteins (Hopkins 1930), and they have also been found to inactivate many bacteria and bacteriophages (Burnet 1933). Precise measurements on the effect of urea on solutions of these viruses have not been made, but it may be said that they are unexpectedly resistant. They retain the ability to give spontaneously birefringent solutions in the presence of saturated urea for some hours, and several hours' exposure is necessary before any considerable part of the protein is denatured. This denatured protein is still largely soluble in water.

Nitrous Acid

It has been shown (Bawden and Pirie 1936; Bawden, Pirie and Spooner 1936) that nitrous acid destroys the infectivity of potato virus "*X*" preparations without affecting their serological activity. Stanley (1936*b*) has described a similar effect on tobacco mosaic virus and we have confirmed this.

Treatment with 15 % acetic acid and 7 % sodium nitrite for half an hour at 0° C. reduces the infectivity without affecting the serological titre. More vigorous treatment causes complete loss of infectivity, but there may also be some denaturation with consequent loss of serological titre and phosphorus. Treatments which just inactivate without denaturation do not affect the serological activity, the phosphorus content, or the ability of the preparations to give spontaneously birefringent solutions. Tobacco mosaic virus preparations inactivated by nitrous acid appear to be completely unaltered antigenically, for tobacco mosaic antiserum absorbed at the optimal combining proportions with non-infective nitrite-treated tobacco mosaic virus will no longer precipitate with fully active virus.

Papain and Clupein

It has already been stated that papain has no enzymic effect on the purified virus preparations, but there is an interesting reaction between a constituent of commercial papain preparations and the purified viruses. When crude papain is extracted with water and the clear extract dialysed it is found that about one-tenth of the original material is indiffusible, and that this fraction retains the enzymic activity. A solution of this indiffusible material precipitates any of these virus strains from dilute neutral solutions, and the precipitate obtained resembles closely that obtained with acid or ammonium sulphate. Examined microscopically with dark ground illumination it has the same "crystalline" or fibrous structure. When varying amounts of papain were added to a constant amount of virus in one experiment it was found that the supernatant fluid, after centrifuging, gave a precipitate with neither papain nor virus when the two were mixed originally in the ratio of 1 of papain to 4 of virus. A similar, but amorphous, precipitate separates when papain is mixed with solutions of yeast, thymus or virus nucleic acids. From these precipitates the active enzyme may be recovered by extraction with acid. With the papain-virus precipitate the extraction should be made at *p*H 3·3, and with the yeast and thymus nucleic acid precipitates at *p*H 1·5.*

The precipitation of papain with both nucleic acids and the virus suggested that the protamines, which are well known to precipitate nucleic acids, might also precipitate the virus. It was found that a neutral solution of clupein sulphate will precipitate any of these virus strains at neutrality, and the precipitate does not become soluble until the *p*H is raised to 8·5 or 9. The virus, however, can be recovered readily by extracting the clupein from the precipitate with phthalate buffer at *p*H 3·3. When recovered in this way the virus has the usual phosphorus content, infectivity, serological activity and ability to give a spontaneously birefringent layer. The supernatant fluid from a mixture of virus and clupein sulphate contains neither substance when their initial ratio in the mixture is 20 to 1. When examined microscopically the virus precipitate with clupein resembles closely that produced with papain.

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* The activity per milligram of the papain that has been extracted from a nucleic acid precipitate is greater than that of the original material. It is possible, therefore, that this process may be useful for the purification of papain.

The precipitates of all three virus strains with either papain or clupein are soluble in dilute salt solution, but there is a definite difference in the amount of salt necessary for the resolution of the precipitates with the different strains. The precipitate with aucuba mosaic virus does not occur if the concentration of sodium chloride is approximately M/20, but with tobacco mosaic virus and Enation mosaic twice this concentration is necessary to inhibit the precipitation. The precise values depend on the concentration of the two reagents and on the temperature, but the distinction between aucuba mosaic virus and the other two strains is clearer here than with any other physical or chemical properties that we have examined.

For a number of reasons these precipitates are of interest. They supply us with other methods for the isolation of these viruses, and possibly of other viruses and bacteriophages as well. For this purpose the precipitates with papain would probably be the more useful, for protamines give precipitates with a number of normal proteins (Lissitzin and Alexandrowskaya 1933). The fact that these insoluble complexes occur suggests that in making extracts from infected leaves some part of the total virus may be lost in the leaf tissue and in the cell debris. Finally, the phenomenon offers a clue to the possible nature of cell inclusions in virus-infected plants and animals, for protamines and histones are known to be constituents of most types of cells.

Effect of X-rays

Gowen and Price (1936) have shown that the infectivity of material obtained by drying clarified sap from tobacco mosaic plants can be destroyed by exposure to X-rays. The amount of virus inactivated was proportional to the amount of irradiation. We have obtained similar results with our purified virus preparations. These have been irradiated both as solutions and as dry powder with the same results. In one experiment with dry material, a 1 mm. layer had its infectivity reduced to about one-hundredth by 3 hr. irradiation, 8 cm. from a copper anticathode of an X-ray tube run at 30 kV and 20 mA. After 7 hr. exposure it was no longer infective. The type of inactivation resembles that following treatment of the virus with nitrous acid, for even after infectivity of the preparations is completely destroyed they still retain their serological activity. Also, when liquid crystalline solutions of the virus are irradiated these are unaltered in appearance and still birefringent when non-infective.

THE STATE OF THE VIRUS IN THE PLANT

The X-ray measurements made on the purified virus preparations show that the individual particles have a constant cross-section area of not less than 20,100 sq. A. It is not known whether the particles also have a constant length, but the extreme character of the orientation phenomena and the X-ray data indicate a minimum length of at least ten times the width. We have no evidence that particles of this length occur in the infected plant. Indeed from the results of experiments comparing the optical properties, activity and filterability of crude infective sap with those of solutions of purified virus it is more probable that the virus is in a different condition in the clarified infective sap, and that in the isolated product it has become aggregated.

The amount of anisotropy of flow shown by clarified sap varies somewhat with different samples, and it seems to be rather more definite with sap from plants grown in the winter. Although the yields of virus isolated from infected plants show that the infective saps must contain from 1 to 2 g. of virus per litre, yet the anisotropy of flow shown by clarified infective sap is usually much less than that shown by a 0·1 % aqueous solution of the isolated virus. It is also much less than that given by a 0·1 % solution of isolated virus in either clarified healthy tobacco sap or clarified infective sap, and the difference, therefore, is clearly not caused by the presence of protective colloids in the sap.

The relative activities of two samples of crude infective sap (or of two similarly treated solutions of isolated virus) are found to be the same whether they are compared for their serological titres or for their ability to produce local lesions, i.e. if one has twice the precipitation end-point with antiserum it will also be approximately twice as infective. When, however, a sample of crude infective sap and a solution of isolated virus giving the same serological titre are compared by the infection method the crude sap is found to be several times more infective. The results of three experiments comparing the serological activity and the infectivity of crude infective sap and 0·1 % aqueous solutions of purified tobacco mosaic virus are given in Table VIII.

The filtration end-points of the virus before and after purification are also widely different. Smith and Doncaster (1936), using crude infective sap, obtained infective filtrates through collodion membranes with an average pore size of 53 mμ. This suggests a particle of diameter 17 mμ, or 170 A, which agrees well with the X-ray measurements of the width of the particles in our purified preparations. The purified virus preparations give

FIG. 2

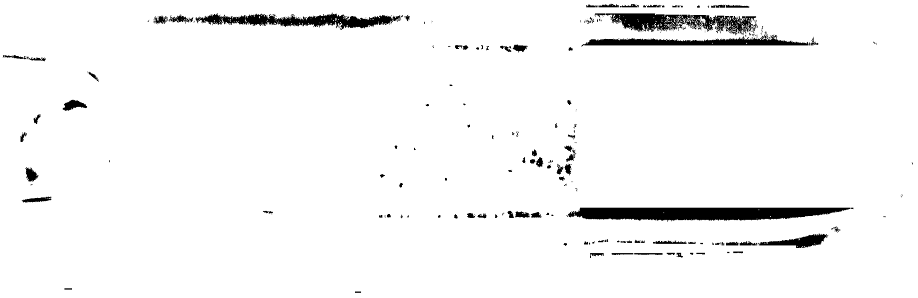

FIG. 1

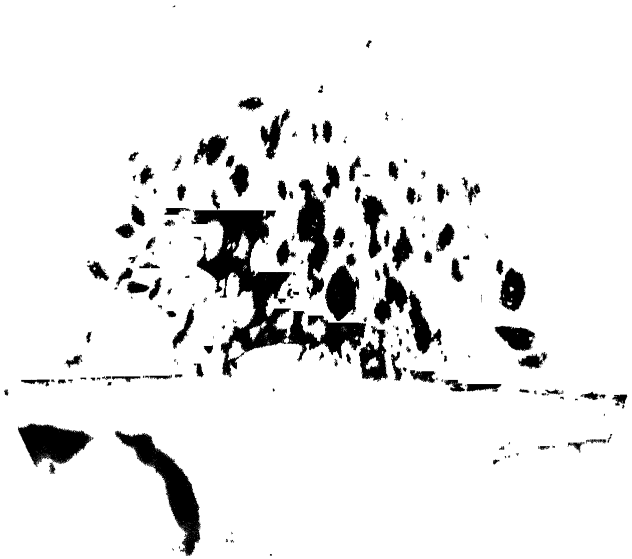

Fig. 3

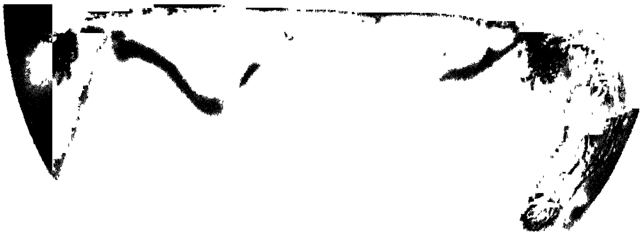

Fig. 4

very different results. In aqueous solution at about *p*H 7 purified virus will not filter through Seitz pads or through collodion membranes with a pore size as great as 450 mμ; the filtrates through such membranes were protein-free, serologically inactive and non-infective.

TABLE VIII—COMPARISONS OF THE SEROLOGICAL ACTIVITY AND INFECTIVITY OF CRUDE INFECTIVE SAP AND 0·1 % SOLUTIONS OF PURIFIED TOBACCO MOSAIC VIRUS

Virus preparation	Serological titre	Infectivity on *Nicotiana glutinosa* at various dilutions Average no. of lesions per half-leaf				
		1/10	1/100	1/1000	1/10,000	1/100,000
Crude sap	1/4000	180	88	45	4	0·8
Purified	1/8000	88	26	13	2	0·6
Crude sap	1/4000	255	168	75	25	5
Purified	1/10,000	208	118	27	5·3	1
Crude sap	1/1000	247	99	34	8	0·8
Purified	1/5000	139	46	17	2·6	0·6

All these results seem to indicate that in the purified virus preparations the individual particles are larger than those in expressed infective sap. Aggregation of the particles end to end would increase the ease with which they are orientated by streaming and so increase the amount of anisotropy of flow shown by a given amount of virus. It is to be expected that such an aggregation would affect also the filterability and infectivity, but not the serological titre to anything like the same extent.

The whole process of purification that we have described is not necessary to produce this apparent aggregation. After one precipitation of the virus from crude sap, with either alcohol, acid or ammonium sulphate, the amount of anisotropy of flow is definitely increased and the filterability is altered. Filterability and the amount of anisotropy of flow are closely correlated, increase in the latter being always accompanied by a decrease in the former.

The aggregated virus cannot be returned to its original condition by putting it into expressed plant sap. When added to clarified healthy sap the purified virus preparations still show quite definite anisotropy of flow, will not filter and their infectivity is not increased. Attempts to render the isolated virus more filterable by dissolving it in clarified infective sap also failed. Indeed, from the results given in Table IX it can be seen that the presence of the purified aggregated virus prevented the normal virus, which had not been precipitated, from passing the filters. In this experiment two samples of tobacco mosaic virus were filtered through a membrane of average pore size, 450 mμ, approximately nine times the size normally required to stop tobacco mosaic virus. Sample 1 consisted of 5 c.c. of clarified infective sap plus 5 c.c. of phosphate buffer at *p*H 7, and sample 2 of 5 c.c. of clarified infective sap plus 5 c.c. of buffer containing 20 mg. of isolated virus. These were tested both before and after filtration for their infectivity and their serological activity.

TABLE IX—EFFECT ON THE FILTERABILITY OF VIRUS IN CRUDE SAP BY ADDING ISOLATED VIRUS

	Serological titre	Infectivity on *Nicotiana glutinosa* No. of local lesions at two dilutions 1/100	1/1000
Clarified infective sap:			
Before filtration	1/320	419	78
After filtration	1/320	390	71
Clarified infective sap plus isolated virus:			
Before filtration	1/25,600	872	426
After filtration	1/20	33	2

Description of experiment in text.

We thus have little evidence as to the shape of the virus particles as they exist in the plants. It seems probable, however, that they have a width of about 17 mμ, and, as infective sap shows some anisotropy of flow, that the smaller units in the plant are either rods or plates or that in the plant a certain amount of aggregation into rods must occur.

In the section on the behaviour of the virus in a centrifugal field it was shown that birefringent jellies could be sedimented from clarified infective sap. Wyckoff, Biscoe and Stanley (1937) centrifuged both clarified infective sap and purified virus preparations, and found that the purified virus had the same sedimentation constant as the heavy constituents of the infective sap. At first sight this may seem to contradict our hypothesis that the particles become aggregated during the processes of purification, for larger particles would be expected to sediment more rapidly. If the particles were spherical this would be so, but no theoretical treatment of rod-shaped particles has yet been made to indicate the magnitude of differences which might be expected in the sedimentation rates of rods of the same width but of different lengths. In other words, it is unknown if the length of rods at all affects the sedimentation rate. That the observed sedimentation rate of the virus is the same after purification as it is before does not mean that the particles are necessarily the same size or that linear aggregation has not occurred, but the fact might equally well be advanced in support of the view that the sedimentation rate of rods is relatively independent of their length.

[*Note added in proof* 19 *June* 1937.—We have found recently that the amount of anisotropy of flow shown by clarified infective sap can be greatly increased by centrifuging for from 2 to 3 hours at 14,000 r.p.m., and then resuspending the sediment in the supernatant fluid. This fact suggests that aggregation occurs as a result of high-speed centrifugation. Centrifugal data on untreated sap, therefore, is of questionable value as an indication of the size of the virus particle, unless it can be shown that the aggregation takes place only in the sediment and not during sedimentation.]

STUDY OF THE SAP FROM UNINFECTED TOBACCO PLANTS

We have been unable to isolate from uninfected tobacco or tomato plants any liquid crystalline proteins similar to those in our purified virus preparations. The protein content of clarified healthy tobacco sap may be only one-tenth to one-fifth of that of infective sap, and what protein there is precipitates differently. If the whole purification process described for the isolation of the viruses is carried out on healthy sap nothing is obtained.

The saps expressed from healthy and infected plants of the same age resemble one another closely both before and after clarification. When either acid or alcohol is added to the clarified saps both give precipitates. The precipitates, however, differ, for those from uninfective saps are darker in colour and only a small portion can be dissolved in water at neutrality. This soluble material is not precipitated by one-third saturation with ammonium sulphate, and the precipitate obtained with acid has not the characteristic satin-like sheen of the virus-containing precipitates. After long contact with ammonium sulphate the solutions often give a precipitate with a slight sheen, but this is merely calcium sulphate.

Both healthy and infective crude saps when heated to 70° C. give flocculent precipitates which aggregate rapidly and are easily removed by centrifuging. The supernatant fluids from healthy sap now contain very little material that can be precipitated with ammonium sulphate and contain only traces of indiffusible phosphorus, but the supernatant fluids from similarly heated infective saps contain the virus.

Healthy sap seems to vary from sample to sample more than infective sap, and we have sometimes found it impossible to isolate the nucleoprotein described below. When large amounts (up to two-thirds saturation) of ammonium sulphate are added to clarified healthy sap a precipitate containing about 1 % of phosphorus often separates. This material (apparently a nucleoprotein, for it gives the protein colour reactions and contains phosphorus and carbohydrate) is quite soluble in half-saturated ammonium sulphate solution; it can be precipitated by acids and does not diffuse through cellophane membranes. Attempts have been made to exclude the possibility that this nucleoprotein may be a contaminant of our purified virus preparations and so responsible for their nucleic acid content. When the nucleoprotein from healthy plants is added to solutions of the isolated virus, the virus can be recovered alone quite easily by precipitation with dilute ammonium sulphate solution. The healthy plant nucleoprotein remains in the supernatant fluid, from which it can then be recovered quantitatively by precipitation with more concentrated ammonium sulphate solution. Unless, therefore, the two combine in a definite ratio it is improbable that our virus preparations are mixtures.

The nucleoprotein found in healthy tobacco plants is much less stable than the virus, and it has often disappeared entirely during the course of a few precipitations with acid or ammonium sulphate, and all the phosphorus has then been found in a form that is soluble in both acid and fully saturated ammonium sulphate solution. When heated to 90° C. the plant nucleoprotein, like the virus, breaks down, and gives a nucleic acid similar to that derived from heat-denatured virus. Proteolytic enzyme preparations that do not affect either the activity or the phosphorus content of the virus preparations readily destroy this normal plant nucleoprotein. This is further confirmatory evidence that our preparations are not mixtures of virus and a normal plant constituent, although it cannot be taken as proof.

We have, therefore, some evidence that this healthy plant nucleoprotein is not merely mixed with virus in our preparations, but no evidence that they are not combined. Our only evidence that the nucleic acid found in our preparations is an integral and essential part of the virus is the constancy of the observed phosphorus and sugar content of a large number of different virus preparations from a number of different host plants, and the fact that as yet we have been unable to get a fully active virus preparation that is phosphorus-free.

It is tempting to speculate on the possible part that this plant nucleoprotein may play in the multiplication of the virus, and to wonder if it is in any sense a precursor of the virus or whether it is one of the constituents of the healthy plant from which the virus is built up. There is no positive evidence for this belief, but the fact that a similar nucleoprotein has not yet been isolated from the sap of infected plants does perhaps favour it.

In addition to the differences in their protein content, the optical properties of saps from healthy and from tobacco mosaic plants also differ. When clarified infective sap is made to flow between crossed Nicol prisms it usually shows some anisotropy of flow, as was first recorded by Takahashi and Rawlins (1933*a*). We, however, have been unable to confirm their later statement that the sap from healthy tobacco plants shows a similar phenomenon (1933*b*). The amount of anisotropy of flow shown by infective sap can be much increased by precipitating the virus with alcohol or acid. The eluates from precipitates obtained by adding acid or alcohol to clarified healthy tobacco sap, however, show none.

DISCUSSION

Many of the implications of this work have already been discussed at some length. Of these, perhaps the most important are our justification for calling our isolated nucleoproteins "viruses" and the relationship between these products and the virus as it occurs in the plant. We may repeat that nucleoproteins with the characteristic optical properties which we have described have not been isolated from healthy plants, and no evidence has been obtained that virus activity can be dissociated from them. Also, the particular protein isolated is dependent entirely on the virus strain used and not at all on the host plant. This is strong presumptive evidence of the identity of our products, although not conclusive. It is, however, extremely difficult to account for the different behaviour of the virus before and after purification unless it behaves in the same manner as the isolated nucleoproteins. Stanley described the product he obtained by precipitation with ammonium sulphate as "a crystalline protein possessing the properties of tobacco mosaic virus". During the processes of purification, however, the virus undergoes a change that is not readily reversed, and loses completely the property that first distinguished tobacco mosaic virus as a new type of disease agent, namely, that it should pass fine filters. Also, the infectivity of the virus is considerably reduced. These phenomena can readily be interpreted if we assume that the virus particles have become aggregated linearly during the processes of purification. The behaviour of our nucleoproteins in solution, and the X-ray measurements, show that they are long particles such as would be produced by this type of aggregation. Further, their width is very similar to that found for the virus in crude sap by filtration experiments.

Since Stanley first described his "crystals", phrases such as "lifeless molecules" have been increasingly applied to viruses, and much has been made of the idea that there is an essential incompatibility between the living and the crystalline states. As this is obviously an aesthetic rather than a scientific incompatibility it is necessary to be clear about the aesthetic connotations of the word crystalline. We have already suggested that the virus "crystals" might more accurately be described as fibres, and we doubt whether it is profitable to say that these viruses can be crystallized, or to apply the word crystalline to them without some qualification. Writers who find this incompatibility usually assume that a crystalline material must consist of a single definable chemical substance; this, however, is by no means necessarily true, for whenever groups of superficially similar substances are studied mixed crystals are found. The proteins form such a group, and, although it has been shown that the haemoglobins often do not form mixed crystals, the point has been so inadequately studied that it cannot be asserted that even true protein crystals are necessarily homogeneous. Structures such as plant fibres, hairs and muscle are fully as crystalline as the solid virus preparations have yet been shown to be, and the organization in suspensions of rod-shaped bacteria (or even shoals of fish) closely resembles that in the liquid crystalline virus preparations. A state of organization that is often described as crystalline is necessarily taken up by any collection of rods of equal cross-section when flowing or when packed tightly, and such states are widespread in nature (Schmidt 1924). Using crystallinity in this sense there is obviously no incompatibility between the living and the crystalline states, and it is only in the sense that any regularity in the arrangement of particles gives that arrangement some of the attributes of a crystalline substance that this term can be applied to the virus preparations. It is, however, unprofitable to attempt to apply the words living and dead to viruses, and here we are confronted with the wider question, which has been discussed elsewhere (Pirie 1937), of whether these words have any rigidly definable scientific meaning at all.

The X-ray measurements on soluble virus aggregates show that there is a regular arrangement of groups along each particle, and the sharpness of the reflexions shows that these particles are sufficiently long for a large number of repeat units to be found in each. Each particle has an internal regularity of the type sometimes found in large molecules, but with the viruses it is on an unusually large scale. In this sense the particle resembles an animal or plant fibre and may be spoken of as crystalline. When the soluble virus aggregates are aggregated still further by acid, ammonium sulphate or clupein, microscopically visible rod-shaped particles are formed. Suspensions of these visible rods show anisotropy of flow at even greater dilutions than solutions of the purified virus. This suggests that the constituent rods of aggregated virus lie parallel in the "crystals", but there is no evidence that adjacent chains of particles bear any relationship to one another as they would in a true crystal. The arrangement in the visible virus aggregates may perhaps be compared with that of matches shaken to the side of a drawer, and the arrangement in a crystal with that of bricks in a wall. There is, as yet, no positive evidence in favour of either arrangement in the virus aggregates, and until evidence is obtained it is probably advisable to avoid the word crystalline in describing solid preparations of the tobacco mosaic viruses.* We see no reason why particles possessing many of the attributes of organisms should not pack into a three-dimensional lattice, and can see no essential incompatibility between crystalline states and those which could be described as living, but only a two-dimensional regularity has been demonstrated as yet in our virus preparations.

Virus preparations denatured by heat break down to give a nucleic acid and some denatured proteins, but no diffusible constituents have yet been observed. Because of this we described our products as nucleoproteins and, except for their capacity for linear aggregation, the description satisfies their chemical and physical properties. We have already stated that after denaturation by heat only protein and nucleic acid have been found. The apparent absence from these viruses of diffusible constituents separates them sharply from the bacteria. A further point of difference is the apparent absence of water from the interior of the virus particles even when they are in solution. It is not the fact that these viruses can be dried without losing all their activity that distinguishes them, for this is a property possessed by some organisms, but it is the fact that they normally occur with no internal water that is unusual. The X-ray measurements already published (Bawden and others 1936) show that the intramolecular spacings are the same whether the measurements are made on dilute virus solutions or on dried films of virus. Only the intermolecular spacings alter, and these are a function of the concentration of the virus solutions. From these results it follows that the internal composition of the individual particles is the same whether the particles are dissolved or in a dried film, for any picture that we may form of a structure able to expand sufficiently to contain an amount of water comparable with that usually found in bacteria, without in any way affecting the distance between the groups causing the X-ray reflexions, is too improbable to be taken at all seriously at present. The individual virus particles, therefore, seem to be solid masses of protein with no interstices, and this interpretation agrees well with the observed specific gravity of the material. In the matter of water content the particles much more nearly resemble protein molecules than they resemble either bacteria or the crystals of those proteins, such as the albumins, which have an open structure.

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* [*Note added in proof* 10 *June* 1937.—Bernal and Fankuchen (1937) now state definitely that the solid preparations of virus as yet studied are not true three-dimensional crystals.]

The fact that the individual particles contain no water makes it difficult to understand what forces hold apart the particles in the jellies which separate when the virus solutions are centrifuged at high speed, for, although they all have similar appearances, the jellies can have solid contents varying between 10 and 30 %. Regardless of the initial solid content, these jellies when diluted with water all give solutions that cease to be spontaneously birefringent at about the same concentration. From this fact we may argue that the constituent particles in all the jellies are of about the same length, by analogy with Staudinger's (1932) treatment of the behaviour of suspensions of rods at different concentrations. Staudinger has found that the graph relating the viscosity of a suspension of rods with its concentration has a sharp break at a concentration at which the rods have insufficient space for free rotation. It is reasonable to assume that in dilute solutions the anisotropic virus particles have sufficient room to move freely in all directions, and therefore are distributed at random. In bulk such dilute solutions will therefore be isotropic unless the random arrangement is interfered with by such forces as shearing. In virus solutions of a certain concentration, however, the virus particles are so close together that rotation about the two shorter axes becomes impossible, although translational motion is relatively unimpeded. At this concentration the fluid will consist of a three-dimensional mosaic of regions arranged at random to each other, but in each of which all the rod-shaped particles will lie approximately parallel. The concentration at which the isotropic virus solutions will become anisotropic (or top-layer fluids will become bottom-layer fluids) will depend on the length of the rod-shaped particles. It also depends on other factors, for impurities, such as virus breakdown products, can impede greatly the separation of the liquid crystalline layer.

When a layer of spontaneously birefringent virus solution is examined with a lens or a microscope through crossed Nicol prisms, the regions of parallel orientation are seen as coloured areas of different hues, for the colour given depends on the path difference between the ordinary and the extraordinary ray in each region, and this in turn depends on the thickness of the region and on the angle that the direction of the rods makes with the plane of polarization. The size of the regions of parallel orientation varies inversely with the virus concentration. The reason for this is unknown, but with a little practice it can be used to estimate the concentration of bottom-layer virus solutions with some accuracy.

If Staudinger's theory can be applied to these virus preparations it is possible to obtain an estimate of the length of the rods into which the virus is aggregated during the processes of purification. At the critical concentration for the formation of a spontaneously birefringent virus solution the ratio of the volume occupied by the virus to that occupied by the water should equal the ratio of the volume of the rod to the volume in which the rod can rotate freely. The volume in which it can rotate freely cannot be less than that of a flat cylinder the diameter of which is the length of the rod and the depth of which is the width of the rod; and it cannot be greater than the volume of a sphere the diameter of which is the length of the rod. The X-ray measurements show that each rod has an effective diameter of 152 A and an area of 20,100 sq. A in the dry state, and we have already suggested that the area is unchanged by dissolving. For tobacco mosaic virus the critical concentration at which a solution becomes spontaneously birefringent is 1·6 %, and as the specific volume of the virus is 0·73, there are sufficient data to calculate the length of the rods. If the length of the rods be taken as $2L$, and if the volume required for the free rotation of the rod be a cylinder, then

$$\frac{100-(1{\cdot}6\times 0{\cdot}73)}{1{\cdot}6\times 0{\cdot}73}=\frac{152\times \pi L^2}{20{,}100\times 2L},$$

while if the volume required for free rotation be a sphere, then

$$\frac{100-(1{\cdot}6\times 0{\cdot}73)}{1{\cdot}6\times 0{\cdot}73}=\frac{\frac{4}{3}\pi L^3}{20{,}100\times 2L}.$$

The two values of L that satisfy these equations are 7000 and 890, giving the length of the rods as 14,000 and 1780 A. It is obvious that these figures can be used only to give the order of magnitude of the particle length, for a large number of assumptions have been made in the calculation. Staudinger argues that his solutions turn from the sol to the gel state when the volume available for each rod is much less than the volume of a flat cylinder. If we adopt this view, then the aggregated virus particles will have a length of 14,000 A, and the reason for their not passing a membrane with pores of 4500 A in diameter is readily understood.

All the layering and other phenomena have been studied in neutral aqueous solutions. As the isoelectric point of the viruses is considerably on the acid side each rod will carry at *p*H 7 a large number of negative charges, for the apparent equivalent weight is only 4000, whereas a particle with a length of 14,000 A and a cross-section of 20,100 sq. A would weigh about 200,000,000 times as much as a hydrogen atom. The fact that the particles are so highly charged may account for the rigidity of the jellies which are sedimented when the virus solutions are centrifuged at high speed, and explain why the virus particles cannot be packed tightly by centrifuging. The large charge also seriously complicates a theoretical interpretation of the critical concentration at which the virus solutions become spontaneously birefringent.

The individual strains of tobacco mosaic virus cause quite characteristic symptoms in plants and it is obvious that they must differ from each other, but as yet we have noticed very few clearly defined distinctions. Of these, the difference in the solubility of the virus-clupein precipitates is the most definite. Bernal will report elsewhere that the three strains we have used give an X-ray pattern of identically spaced lines, but the intensities of the individual lines differ with the different strains. Wyckoff, Biscoe and Stanley (1937) state that aucuba mosaic virus has a sedimentation rate greater than tobacco mosaic virus and deduce from this that it has larger particles. This is difficult to correlate with our results. The X-ray measurements show that the two strains have the same area of cross-section and shape, and we have been unable to produce spontaneously birefringent solutions of aucuba mosaic virus as dilute as those of tobacco mosaic virus. If this property is a function of the length of the particles the observation suggests that the rods of aucuba mosaic virus are the shorter.

The physical and chemical properties and general appearance of solutions of the three strains are very similar. Stanley (1936*a*) in his paper on tobacco mosaic virus made no mention of a sheen in his preparations, but recently (1937) he has claimed that solutions of aucuba mosaic virus show a more pronounced sheen than solutions of tobacco mosaic virus, and that aucuba mosaic virus is more easily precipitated by ammonium sulphate and has an isoelectric point at *p*H 3·7 instead of *p*H 3·4. We are unable to agree with the first statement, for with a large number of different preparations of the three strains we have observed no consistent differences. The amount of sheen noticeable in a solution of given concentration, salt content and *p*H value varies with different preparations of the same strain, and becomes less if the solution is allowed to remain quite still for a long time or if it is centrifuged at high speed. Similarly, the ease with which the viruses can be sedimented in the presence of dilute ammonium sulphate solution varies greatly with different preparations of the same strain, unless the preparation has been subjected to one of the treatments we have described for further purification. Differences noticed in the ease of precipitation with salts require cautious interpretation until the purity of the material used has been much more clearly defined.

The three different strains are closely related serologically, but antigenic differences can be found between them by cross-absorption experiments. These experiments will be described in a later paper.

The means by which these viruses increase in the plant are unknown. Stanley (1936*a*) suggests that the viruses are autocatalysts and cites trypsin and trypsinogen as an analogy. Although this view cannot be disproved there is some evidence against it. No protein has yet been found in healthy plants that is so similar to the virus as trypsinogen is to trypsin. Wyckoff, Biscoe and Stanley (1937) give the molecular weight of the virus protein, calculated from centrifugal data, as greater than 10,000,000, but they could find no particles in healthy sap with a molecular weight of more than 30,000. The amount of soluble protein in healthy tobacco plants is considerably less than that in infected plants. These facts make it improbable that the virus can catalyse the conversion of a normal soluble constituent of the plant into virus. Definite conclusions on the autocatalytic hypothesis can, however, only be drawn when we have much more information about the differences between those constituents of infected and healthy plants which are insoluble in water. It is possible that the virus is built up from molecules which are essential to the normal plant in small quantities; presumably if these were converted into virus the plant would be stimulated to produce more, and in this way the additional soluble protein in the infective saps could be explained. It is tempting to associate the materials from which the virus is reproduced with cell division or growth, for sap from actively growing plants is much richer in virus, and systemic symptoms are usually produced only in those parts of the plants that are growing. There is no positive evidence for this view, but the observation of Sheffield (1936) that mitosis precedes the production of local lesions in *Nicotiana glutinosa*, even when the leaves are mature and cell division has normally ceased, does perhaps support it.

Tobacco mosaic virus is one of the smallest and most stable of plant viruses. It differs widely from most others in many of its properties, and it is doubtful if the results we have described can be at all generally applied to plant viruses. Some presumptive evidence that the activity of potato virus "*X*" is connected with protein has already been described (Bawden and Pirie 1936), but that this protein differs widely from those derived from tobacco mosaic plants is quite evident from their different behaviour in the presence of proteolytic enzymes. From cucumber plants infected with cucumber viruses 3 and 4 we have isolated nucleoproteins similar in many of their properties to those described in this paper (Bawden and Pirie 1937). The cucumber and tobacco mosaic viruses have different host ranges and differ in some of their physical properties. The cucumber viruses have at least one antigen in common with tobacco mosaic, and purified preparations readily show anisotropy of flow and give dilute liquid crystalline solutions. The ability of tobacco mosaic virus preparations to show these phenomena is much more closely linked with their serological activity than with infectivity, for preparations inactivated by heat, drying, acid or alkali lose their serological activity and their ability to give anisotropic solutions, but when infectivity is destroyed by some other agents, such as X-rays and nitrous acid, the preparations still retain their full serological activity and still readily show anisotropy of flow and form dilute liquid crystalline solutions.

We have great pleasure in thanking Mr. E. T. C. Spooner for preparing all the antisera used, Dr. K. M. Smith and Mr. J. P. Doncaster for doing the filtrations through collodion membranes, and Mr. J. D. Bernal for much help and advice.

SUMMARY

Nucleoproteins with characteristic optical properties have been isolated from solanaceous plants infected with three strains of tobacco mosaic virus but not from healthy plants. These proteins are infective at a dilution of $1/10^{10}$ and give specific precipitates with antisera at a dilution of $1/10^{7}$. Solutions of the highly purified proteins separate into two layers if the protein content is raised above about 2%. The lower layer is the more concentrated and is birefringent, while the upper layer shows anisotropy of flow. There is no essential difference in the virus activity, expressed in terms of solid content, of the two layers. The anisotropy of flow can be easily recognized in solutions containing only 0·02% of protein. When centrifuged at high speeds these solutions deposit the protein in the form of a birefringent jelly.

No enzyme preparation has yet been found which attacks these proteins at an appreciable rate, but the activity can be affected by a number of chemical agents.

The stability towards drying and heating has been studied and the conditions under which the nucleic acid/protein complex breaks down.

The physical properties of virus preparations and the X-ray measurements on them are interpreted on the theory that in purified preparations the constituent particles are rod-shaped, and it is suggested that these rods are built up by the linear aggregation of smaller units. There is evidence that, in the plant, part at least of the virus is not aggregated, for filters which pass an infectious filtrate with untreated plant sap do not do so with purified preparations.

The relationship between the nucleoproteins and the viruses is discussed.

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DESCRIPTION OF PLATES

Plate 12

FIG. 1—Photograph, in polarized light, of a 2 % solution of purified tobacco mosaic virus which has been allowed to settle. The lower layer is birefringent, whereas the upper shows only anisotropy of flow.

FIG. 2—Two identical jets of 0·2 % Enation virus solution are flowing into a glass-sided cell 6 mm. deep and full of the same virus solution. The cell has crossed polarizing screens on opposite sides, the plane of polarization of the incident light is horizontal. One jet, *A*, is flowing in the plane of polarization, whereas the other, *B*, is at an angle of 45° to it. The stream *B* shows clearly, whereas the stream *A* is not itself visible but only the turbulence on either side of the actual jet can be seen. From this it is clear that the appearance of a virus solution in polarized light does not give a true picture of the actual motion of the fluid. The actual motion can be deduced from it, however, if we know how the appearance of the individual anisotropic rods varies as they rotate about an axis lying in the plane of polarization. (Natural size.)

Plate 13

FIGS. 3 and 4—Photomicrographs, in polarized light, of the interface between "top" and "bottom" layers in a cell 1 mm. deep. In fig. 3 the photograph was taken after 6 hr. and in fig. 4 after 24 hr. when the separation had become complete. The "bottom" layer is, as described in the text, highly coloured. (Magnification × 8.)

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Analytic Studies in Plant Respiration

VII—Aerobic Respiration in Barley Seedlings and its Relation to Growth and Carbohydrate Supply

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Introduction

Preliminary to a study of seedling respiration as affected by varying concentrations of oxygen, some sets of experiments were carried out in air as a standard oxygen medium. In these experiments growth was also measured and the relation between the rates of these two processes was determined.

The major features of the carbon metabolism in the young seedling are, (1) hydrolysis of starch in the endosperm, (2) formation of new dry matter in the embryo by growth, (3) respiration of carbohydrates to carbon dioxide and water in both endosperm and embryo. By relating (1) with (2) and (3) we arrive at (4) the rate of translocation of carbohydrates from endosperm to embryo. All these activities may be expressed, for numerical comparison, in terms of hexose units per unit of time, so that it is possible to survey, on this common basis, the balance and relative rates of these processes. The rates of the component parts of the system are to be labelled as follows: H, production rate of hexoses by hydrolysis of carbohydrate reserves in the endosperm; R_1, respiration rate of hexoses in the endosperm; T, translocation rate of hexoses from the endosperm to the embryo; R_2, the respiration rate of the embryo; G, the rate of growth of dry weight of the embryo material. We may add to this series P (as a subsection of G) the rate of transitory deposition in the embryo of mobile reversible carbohydrate reserves such as starch, which are later available for respiration. G less P would stand for the irreversible growth of new tissue substance, in a restrictive sense, while G stands for the whole dry-weight increase.

Balancing these rates about the translocation rate we can write:

$$\text{Endosperm} \xrightarrow{\hspace{3em}} \text{embryo}$$

$$[H-R_1] = T = [G+R_2].$$

The study of the CO_2 production of the living combined system gives us the sum of R_1+R_2, while G can be arrived at, up to any moment, by determining the dry weight of the embryo dissected from the endosperm. As it is possible to separate by a special technique of dissection the living embryo-part of the system from the endosperm-part, we can carry out a further analysis and determine separately R_1 and R_2.

Material—Two samples of Spratt-Archer Barley (*Hordeum distichum* var. *nutans*) were used. One was of the 1928 crop, obtained from the University Farm, Cambridge, to be known as (U.F. 1928), and the other, of the 1929 crop, obtained from the Cambridge Seed Testing Station to be known as (S.T.S. 1929). The 1929 sample showed a lower percentage germination (86 %) than the 1928 sample (94 %), as observed at 40–42 hr. after the addition of water at 22° C.

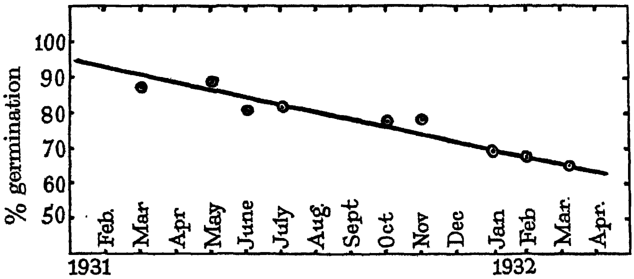

FIG. 1—Drift of germination capacity of S.T.S. 1929 barley with time. The rate of decline in viability is seen to be approximately constant over the period shown, which lasts from the 18th to the 32nd month after harvesting.

The percentage germination yielded by the barley decreased with increasing time of storage. In fig. 1 are plotted the percentages for S.T.S. 1929 barley in the various months of the years 1931–2. There is a pronounced falling off in the germination capacity, the curve being approximately linear. The curve extrapolated back towards the harvest date suggests a hypothetical germination of 100 % in September 1930, a year after harvest. After maintaining this high level for a year, loss of viability sets in, appearing to be a linear function of time.

Apparatus and Method—A form of glass respiration chamber was devised for easy examination of the seedlings during development. This chamber rendered possible the removal, with very little disturbance, of such seeds as showed no signs of germination during the early hours of the experiment.

Each chamber consisted of a large U-tube (fig. 2) of 250 ml. volume placed horizontally in a water-bath maintained at a temperature of 22° C. The U-tube had inlet and outlet tubes for the air and an inlet tube for water. Two glass frameworks of triangular cross-section, covered with muslin to carry the grains, were constructed so as to slide easily in or out of the two limbs of the U-tube.

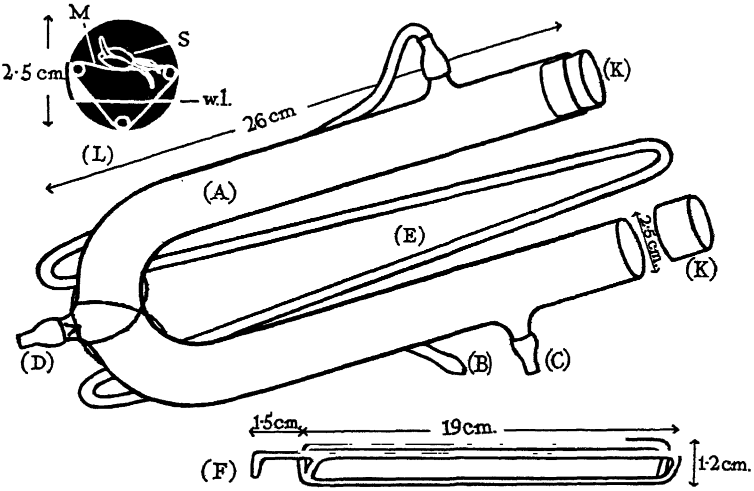

FIG. 2—The horizontal respiration chamber, (*A*) viewed obliquely from above, consisting of a glass U-tube of 250 ml. volume with inlet (*B*) and outlet (*C*) tubes for air and an inlet tube (*D*) for water. The entering air is warmed to the temperature of the water-bath by passing first through a length of glass tube (*E*) bent in zigzag form attached to the chamber.

(*F*) One of the two frameworks made of glass rod designed to fit in the two limbs of the U-tube respiration chamber. It is of triangular cross-section and when covered with a muslin sheath supports the seeds above the level of the water in the chamber. Frames are withdrawn by loosening the rubber bungs (*K*).

(*L*) Transverse section of one limb of respiration chamber showing framework in place with muslin sheath *M*, seeds *S* and water level *w.l.*

Before each experiment the chambers and the frameworks were sterilized, first by boiling and then by dry heat. The dry seeds were placed on the upper stretch of dry muslin and the frameworks slid into the chambers, 100 seeds being placed in each chamber and 40 ml. of water added through the inlet tube giving a level of water a little below the seeds. After intervals varying from 30 to 50 hr. from the addition of water the two frameworks were withdrawn from the chamber for a short period and the grains which had not germinated were picked up with forceps and removed.

All the results in this paper are presented numerically on the basis of the average rate of carbon metabolism of *one germinating seedling multiplied* *by* 100. The mean weight of 100 grains of the University Farm grown barley (U.F. 1928) was 4·593 g. The variation of individual weighings from these means was not greater than ± 0·2 g.

The chambers were darkened during all experiments by covering the top of the thermostat bath with a black cloth. The green shoots of the barley were, however, found to be incapable of appreciable assimilation in the seedling stages used, and removal of the cloth for periods of time had no effect on the course of the respiration.

Measurement of the Respiration Rate—Continuous series of records of the carbon dioxide production of the seedlings were obtained, using aspirators to draw CO_2-free air from outside the laboratory through the chambers and then through Pettenkofer tubes each containing 75 ml. of N/15 baryta. The carbon dioxide absorbed in the Pettenkofer tubes was estimated by titration with N/10 hydrochloric acid. The Pettenkofer tubes through which the gas passed were changed every 3 hr. unless otherwise stated by means of the "air current commutator" in general use in the Cambridge Botany School. The volume of air current passed per 3 hr. was about eighteen times the volume of the chamber, so there should be no injurious accumulation of carbon dioxide in the respiration chambers.

1—Respiration of the Entire Seedling System during Germination

Some sixty experiments were carried out involving the measurement of the respiration rate of the early stages of the germinating barley. Most of the experiments were compounded of periods in air with periods in other concentrations of oxygen. Only nine were carried through entirely in air; these supplied the evidence displayed in figs. 3 and 4 for continuous respiration in air of aggregates of one hundred barley seedlings.

In fig. 4 three experiments with the U.F. 1928 barley are shown as separate curves, while the shaded curve represents the range covered by six S.T.S. 1929 barley curves, four of which are shown in fig. 3 (two being omitted to avoid confusion of the tracks). The approximate appearances of the developing seedlings are shown at different times by sketches along the abscissa axis of fig. 3.

The CO_2 curves from the separate experiments are very similar to each other, showing but little difference between the two samples of barley though of different years and from different sources. For each experiment the curve shows an initial region of slow acceleration of the respiration rate, a middle region of approximately constant acceleration and finally a falling off to zero acceleration as the respiration rate reaches a strikingly well-defined upper limiting value. This slightly undulating upper limit to the respiration rate in the young seedlings is a constant feature and maintained up to the time limits of our experimentation. Presumably, under the conditions of these experiments it would be succeeded by a region of falling respiration rate.

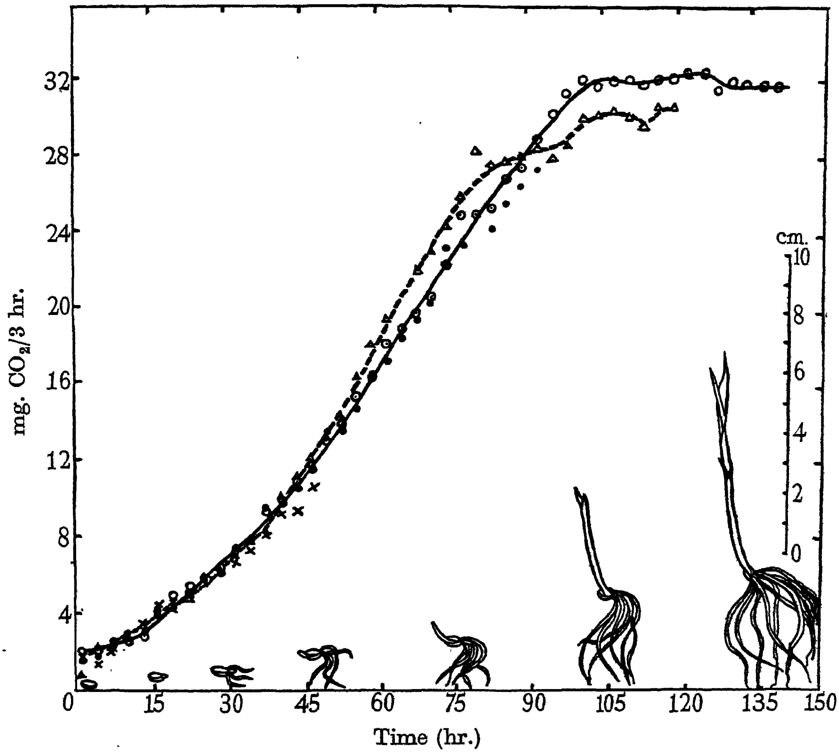

FIG. 3—The course of respiration for 100 germinated seedlings in air during germination of the S.T.S. 1929 barley. The data of four experiments are shown by dots, circles, crosses and triangles respectively. Lines (one continuous, one interrupted) are drawn through two sets of data (circles and triangles). The approximate stages of development and sizes of the seedlings at various times are shown in drawings above the abscissae, with a scale of cm. adjacent.

Differences in Respiratory Behaviour between the two Samples of Barley—Of the two barleys, the S.T.S. 1929 starts at a higher rate than the U.F. 1928 barley but does not accelerate so rapidly, so that the U.F. 1928 curve presently crosses the other and in fig. 4 lies above it in the middle region of the curves. Both barleys ultimately attain the same upper limit rate of respiration of about 32 ± 1 mg. CO_2/3 hr. per 100 germinated grains. In the middle region of the curves the acceleration of the U.F. 1928 barley is 1·31 mg. CO_2/3 hr. per 100 grains (mean of three values), while for the S.T.S. 1929 barley it is 1·25 mg. CO_2 (mean of five values).

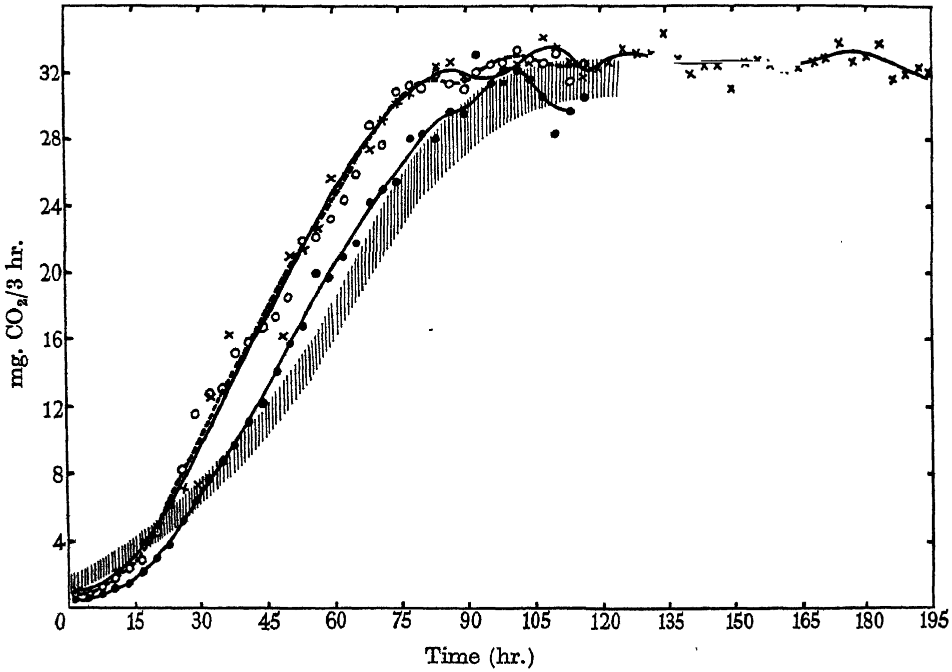

Fig. 4—The course of respiration in air during germination of U.F. 1928 and S.T.S. 1929 barley. Dots, crosses and circles respectively represent the experiments with the U.F. 1928 barley. Continuous lines are drawn through the crosses and dots while an interrupted line is drawn through the circles. The track of the adjacent shaded zone represents the range of divergence of the data obtained in six experiments with S.T.S. 1929 barley (including the four cases detailed in fig. 3). Differences are observed between the two barleys in the initial and middle regions of the curves but the upper limit is approximately the same for both.

Fig. 4 demonstrates the major similarity of the respiratory behaviour of the two samples of the one strain of barley, though it provides also quite definite consistent minor differences between the two samples in the progress of their respiration during germination.

2—THE RESPIRATION OF ISOLATED ENDOSPERMS OF BARLEY

Brown and Morris (1890) experimented upon the growth of barley embryos fixed in contact with dead endosperms that had been kept for months previously in spirit, and decided that the endosperms of graminaceous seeds were merely non-living storehouses of reserves, incapable of exhibiting respiratory activity. They showed that excised embryos will grow on foreign endosperms, on nutrient solutions and on preserved endosperms. Part of their deduction, however, remains open to question: though they showed that embryos will grow on dead endosperms, they did not show that the endosperms of living seeds are dead.

In the present work experiments were carried out, with the U.F. 1928 barley, to determine whether the endosperms of barley grains were capable of respiring by themselves. On removal of the embryos, available supplies of sugar which would have been translocated into the embryo are left in the endosperm and several respiratory possibilities arise:

(*a*) The endosperm will show a high rate of respiration which will be maintained. This would indicate the presence of numerous active centres of respiration in the endosperm and also the continued presence of the enzymes concerned with the hydrolysis of the reserves.

(*b*) The endosperm will show a high rate of respiration at first, but this will fall, indicating lack of enzymes to maintain the supply of sugar from the reserves.

(*c*) The endosperm will show a low rate of respiration which will be maintained over a considerable period. This indicates a low grade of respiratory activity in the endosperm due to the presence of few active centres of respiration.

Method for Separating Endosperms and Embryos under Sterile Conditions—Rigorous precautions were found to be necessary to achieve sterility during the dissection of endosperms from embryos, as infection of the endosperms by bacteria and yeasts took place very easily. A technique was therefore devised to obtain sterile conditions for dissection, as being much preferable to sterilizing with toxic chemical agents.

A chamber, 2 ft. high with a base 20 in. square, was constructed, consisting of a wooden framework covered on the four sides and top with fine-meshed cloth. A glass slab was placed on top of the chamber to prevent accumulation of dust on the cloth surface and a glass window was inserted in the front, sloping inwards above, so that it was possible to see the base of the box while seated before it. The chamber was fitted with electric light. Flaps in the cloth at the front permitted the entrance of hands and arms. The whole chamber was placed in a metal tray lined with blotting paper soaked in glycerol to trap any bacterial or fungal spores which drifted on to it. A small dish of polymerized formaldehyde was placed inside the chamber to ensure an antiseptic atmosphere.

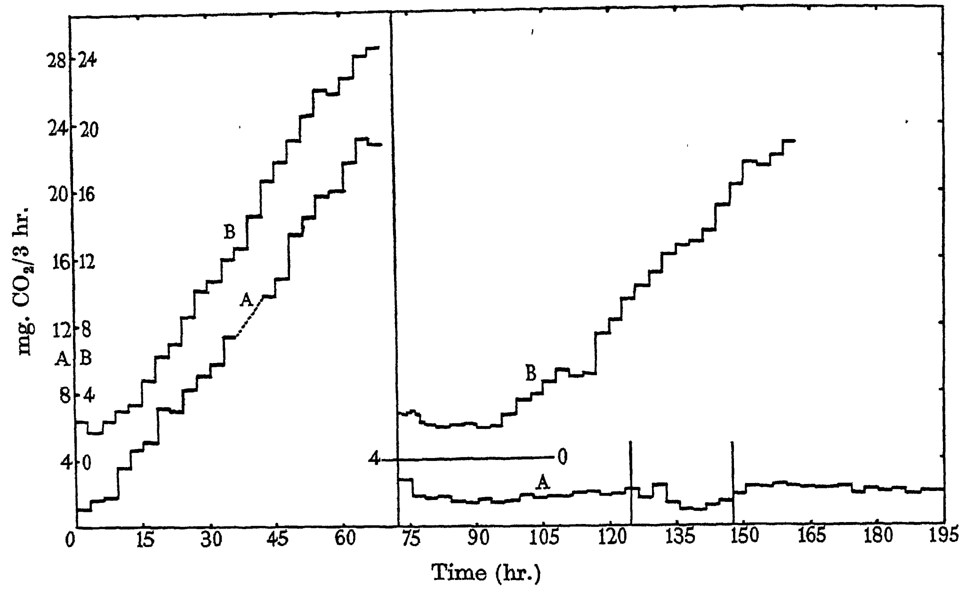

Fig. 5—Respiration of isolated endosperms of U.F. 1928 barley. The data of two different experiments *A* and *B* are shown superposed in the one figure. For clearness two ordinate scales are given, the zero for the ordinates of *B* is displaced by 4 units above that of *A*. Each record begins with the rising drifts of respiration rates of whole seedlings. Dissection of the endosperms took place at about hour 70, after which follow the respiration rates of 100 endosperms. In curve *A* the isolated endosperms remained free from signs of bacterial or fungal infection to the end of the experiment (123 hr. after dissection). Curve *B* shows the steady respiration rate of sterile endosperms for 24 hr. after dissection and then the rate of CO_2 production increasing with the development of infection.

A period of 24 hr. in nitrogen was interpolated during the endosperm respiration shown in curve *A*, but the implications of this will not be discussed in this paper.

For this dissection, seedlings were removed from the respiratory chambers after about 70 hr. of germination when they had obtained a shoot length of 1–2 cm. corresponding to a respiration rate of about 24 mg. CO_2/3 hr. The dissection was carried out with scalpels and forceps sterilized by burning methylated spirits. The operator's hands were sterilized by washing in dilute corrosive sublimate solution. After the dissection the endosperms, alone, were replaced on the frames in the respiratory chambers and the readings continued.

Respiration of Isolated Endosperms—With this technique sterility was completely or largely achieved, and the data from two experiments are exhibited in fig. 5. Curves *A* and *B* exhibit different durations of sterile vitality of the endosperm (see the subscript to the figure).

The respiration rate of the sterile endosperms is seen to be low, 2 mg. CO_2/3 hr. per 100 endosperms, but far from negligible. It remains constant in rate although there is a large mass of carbohydrate stored in its tissues. It seems evident that sugar must be in excess and the number of respiratory centres in the endosperm be the limiting factor of the respiration rate. The respiration system of the endosperm, R_1, takes but a small share of the products of hydrolysis in the endosperm compared with that passing to R_2+G in the embryo (see p. 321).

Presence of Living Protoplasm in the Endosperm Cells—The respiratory behaviour does not suggest a declining residue of embryonic protoplasm left behind but rather a continuing low vitality of the endosperm. Possibly the cells of the endosperm might be in a state of partial autolysis. The work of Lyon (1928) supports the view that the cells of the endosperm contain living protoplasm. This worker found that embryo-less seeds were of fairly frequent occurrence in wheat (0·1 % embryo-less) and that these seeds, when placed under suitable conditions for germination, would respire giving 22·0 mg. CO_2/g. in 6 days at 20° C. This is equivalent to a rate of 1·8 mg. CO_2/3 hr./100 endosperms, assuming 4 g. to be the weight of 100 embryo-less seeds (endosperms). Thus a value for the respiration rate comparable with that of barley endosperms is given by undamaged embryo-less wheat seeds.

3—RESPIRATION OF ISOLATED BARLEY EMBRYOS

That isolated barley embryos can be grown on a variety of artificial media was shown by Brown and Morris (1890). The present experiments were planned to examine the behaviour of isolated embryos supplied only with distilled water. By determining the subsequent drift of their respiration rate under starvation it was hoped to obtain information regarding the grade of utilization of translocated products of endosperm hydrolysis within the embryo. Such products may be utilized in (1) growth of new tissues ($G-P$), (2) as deposits of carbohydrates (P) or (3) in respiration (R_2). The form of the respiration curve of the isolated embryos might give information regarding the magnitude of (2) within the young embryo.

Method—The U.F. 1928 barley was used for these experiments. The dissection in each case was carried out when the seedlings had a shoot length of about 1 cm. and had attained a respiration rate of about 20 mg. CO_2/3 hr./100 seedlings. The use of the sterile dissecting box was not needed as the isolated embryos apparently constitute a very scanty source of material essential for the growth of fungi and bacteria, and infection was never apparent.

The "Starvation" Curve of Embryo Respiration—The form of the drift of the respiration rate of the isolated embryos is shown by the typical record presented in fig. 6. Two further experiments (not presented) gave similar curves but in each of these cases the record was interrupted by supplying nitrogen in place of air for 12 hr. periods at various stages. The data so obtained will not be considered here. The record in fig. 6 shows typical features of starvation and senescence. The only supply of carbohydrates for the respiration and growth of the embryos is that present in the tissues at the time of excision. The rate of respiration falls greatly on isolation and we assume the fall is due to decrease of supply of the respirable substrate. In this starvation state the respiration rate falls at first rather steeply; later

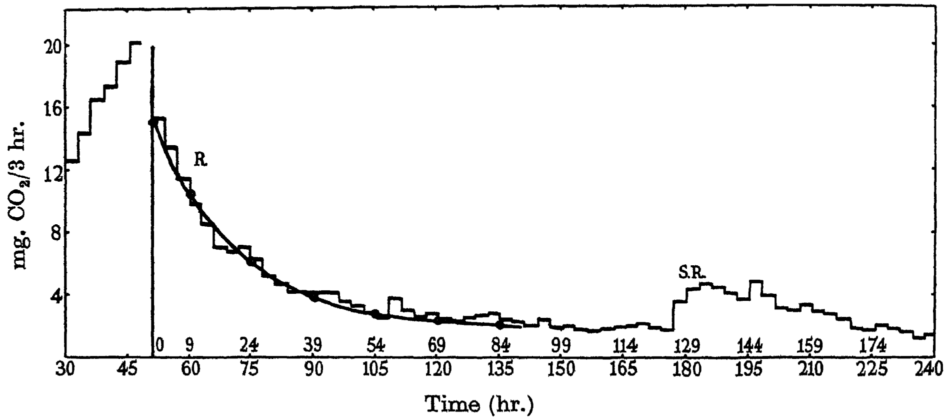

FIG. 6—Respiration of isolated embryos of U.F. 1928 barley. This record begins with the respiration drift of whole seedlings, followed, after dissection, by the respiration drift (*R*) of 100 isolated embryos. The curve of embryo respiration shows typical features of starvation and senescence (*S.R.* = "senescent rise"). The smooth form of the starvation respiratory record suggested that a simple curve might be fitted to it. The logarithms of the excess respiration rates above the low limiting value of 1·8 mg. finally obtained were found to give a straight line. On further analysis the type of curve suggested for the succession of *R* values would be given by $R = \alpha e^{-ct} + R_\infty$. It was found that the values for the constants of this curve were $\alpha = 13{\cdot}2$, $c = 0{\cdot}048$ and $R_\infty = 1{\cdot}8$. The curve from this equation is shown as a continuous line in the figure.

the curve flattens out and approaches asymptotically a minimum respiration value of 1·8 mg. CO_2. After 30 hr. at this value a senescent rise occurs about 130 hr. after excision of the embryos. For the starvation part of the record we suggest that the simplest picture of the carbohydrate metabolism is that given in Fig. 6.

At the time of dissection there is a definite amount of the sugar substrate present. In the excised embryo sugar continues to be produced from starch (or other reserve carbohydrate) at rates less rapid than those at which it is consumed in respiration. This production-consumption balance comes to equilibrium at the low value of 1·8 mg. CO_2/3 hr., when the rate of respiration is pictured as being equal to the rate of hydrolysis of starch.

These features suggest that, in the rapidly growing embryo, the magnitude of the carbohydrate reserve is not large and hence we may deduce that the rate of deposition of reserve in the embryos, P, is small in relation to the other fates of sugar.

The Senescent Rise—The rise (*S.R.* in fig. 6) which follows the asymptotic level of the respiration rate may be interpreted as an effect of senescence resulting from the lowering of the "organization resistance" of the cells of the embryos. The increased respiration rate is, presumably, due to the lowering of the resistance to the interaction of the enzymes concerned in the process and their substrates. The falling respiration rate following the maximum may be due to falling sugar substrate concentration or to enzyme deactivation.

Effect of Sucrose Solution on the Respiration Rate of Isolated Embryos—In order to confirm that the falling curve for the respiration rate of the excised embryos was a "starvation" effect, one experiment was carried out with a 0·5 % solution of sucrose, added to the respiration chamber in place of water 48 hr. after the excision of the embryos. Sucrose was chosen as Brown and Morris (1890) had found it to be the most efficient sugar for promoting growth of excised embryos.

The effect of the addition of sugar is shown in fig. 7, the times of these additions being indicated in the subscript. A rise of the respiration rate occurred and was maintained for some hours before the fall began once more. The height attained did not reach the initial rate of respiration of the embryos and so it is possible that something else, besides sugar, supplied by the endosperm is necessary to obtain that initial rate, or that, alternatively, deactivation of the respiration enzymes had occurred to some extent during the starvation period preceding the addition of sucrose solution.

4—COMPARISON OF GROWTH RATE AND RESPIRATION RATE OF ATTACHED EMBRYOS

The products of endosperm hydrolysis which enter the embryo (expressed as T, see p. 321) are shared between the processes of respiration and growth. A set of experiments with the S.T.S. 1929 barley was carried out to look for any evidence of quantitative relationship between these processes at various stages of development of the seedlings. For each experiment 100 seeds were placed in a respiration chamber and a record of their respiration followed up to a prearranged point of time: the seedlings were then removed from the chamber for the determination of their achieved growth. A set of twelve such experiments was carried out for graded periods of time, from 3 hr. to 162 hr., so as to provide a series of growth-respiration pairs extending over the whole developmental range.

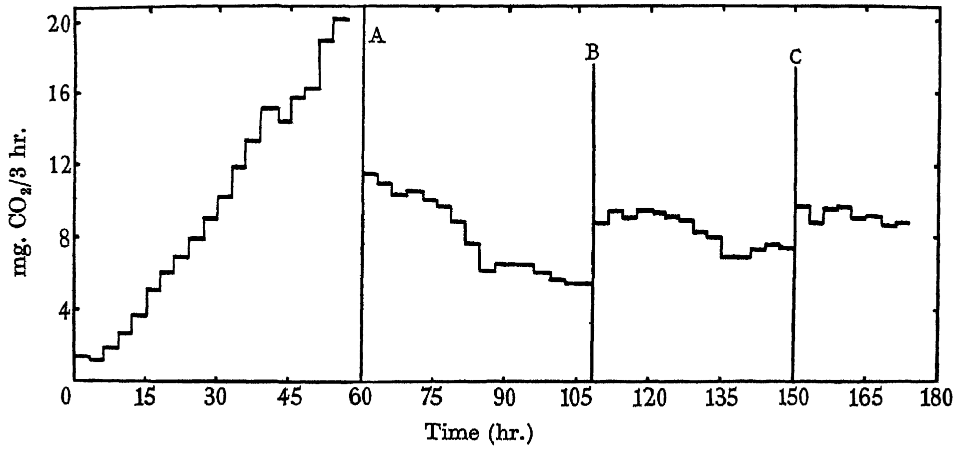

FIG. 7—Respiration of isolated embryos of U.F. 1928 barley. The record begins with the rising curve of respiration rate of whole seedlings, then after the first vertical line *A* the respiration rate drift of the isolated embryos is given. Two successive additions of 40 c.c. of 0·5 % sucrose solution took place at the times indicated by the vertical lines *B* and *C*. In each case the liquid present in the chamber was withdrawn just before addition of the new solution. Rises in the respiration rate of the embryos followed the addition of sucrose in each case.

The primary data of these experiments appear in the twelve lines of Table I, where the results of following growth by several simultaneous indexes are set out in parallel columns, including shoot length, fresh weight of the dissected embryos, dry weight of embryos and dry weight of endosperms. In this table appear also various presentations of the final respiration rates, all in units of mg. CO_2/100 seedlings/3 hr. The respiration directly observed is always that of the whole seedlings while the values for the embryos alone are derived by a constant deduction for the endosperm.

The details of the treatment of the data are set out in the subscript to Table I.

TABLE I—RESPIRATION AND GROWTH DATA OF BARLEY SEEDLINGS

1	2	3	4	5	6	7	8
Time hr.	Seedling respiration mg. CO_2	Mean shoot length cm.	Embryo fresh wt. g.	Embryo dry wt. g.	Endosperm dry wt. g.	Embryo respiration mg. CO_2	Respiration per g. dry wt. embryo
3	—	0	0·241	0·087	3·982	—	—
18	2·6	0	0·373	0·113	3·987	0·6	5·3
48	10·6	0	1·119	0·162	3·758	8·6	53·2
69	19·4	0·72	3·839	0·445	3·441	17·4	39·1
78	26·1	1·25	4·643	0·528	3·509	24·1	45·6
93	29·3	2·34	8·628	0·621	3·106	27·3	44·0
96	29·8	3·29	16·69	0·907	2·752	27·8	30·6
111	34·8	5·21	16·15	1·115	2·373	32·8	28·0
120	30·7	5·58	11·20	1·184	2·280	28·7	24·2
141	31·6	7·70	17·63	1·204	1·703	29·6	24·6
159	29·8	9·80	15·59	1·418	1·776	27·8	19·6
162	33·7	11·42	21·39	1·523	1·430	31·7	20·8

Subscript to Table I

S.T.S. 1929 barley was used. Each line represents the end-state of a separate experiment which was continued for the period of time indicated in col. 1. Col. 1 gives hours from addition of water to the dry seeds to the time when the seedlings were removed from the chambers for dissection and estimations of growth. Cols. 3, 4, 5 and 6 contain data of observed growth; but while col. 3 gives a mean value, cols. 4, 5 and 6 are the sums of weight for 100 individuals. The values of col. 5 are plotted in fig. 8 to give a rising curve of dry weight for the embryos.

Cols. 2, 7 and 8 give respiration rates (mg. CO_2/3 hr./100 individuals) at the end of each experiment, being actually measured for the 3 hr. period ending with the hour set out in col. 1. The embryo respiration in col. 7 is arrived at by deducting from the values of col. 2 a constant allowance of 2·0 mg. CO_2/3 hr. for the respiration of the endosperm. In col. 8 the embryo respiration is presented per unit of dry weight (col. 7/col. 5): these values are plotted in fig. 10.

In Table II are presented derivative values of growth rates, and in this connexion the respiration rates are converted from CO_2 units to hexose units in order to be directly comparable with the carbohydrate units which give the rate of growth. The details of manipulation of the *G* and *R* data are set out in the subscript to the table, the ultimate objective being to arrive at a series of ratios between gain of dry weight in growth and loss of dry weight as respired hexose throughout the development of the seedling. Such ratios appear in the last two columns of Table II.

TABLE II—RATES OF GROWTH AND RESPIRATION OF THE EMBRYO COMPARED FOR BARLEY SEEDLINGS (ALL IN HEXOSE UNITS)

1	2	3	4	5	6	7	8
Time hr.	Dry wt. embryos mg.	Embryo G	Seedling observed R_1+R_2	Seedling interpolated R_1+R_2	Embryo R_2	G/R_2	$G/(R_2+G)$
3	87	—	—	—	—	—	—
(10½)	—	5·2	—	—	—	—	—
18	113	—	1·8	—	—	—	—
(33)	—	4·9	—	4·0	2·6	1·9	0·65
48	162	—	7·2	—	—	—	—
(58½)	—	40·4	—	10·8	9·4	4·3	0·81
69	445	—	13·6	—	—	—	—
(73½)	—	27·7	—	16·0	14·6	1·9	0·65
78	528	—	17·8	—	—	—	—
(85½)	—	18·6	—	19·3	17·9	1·05	0·51
93	621	—	20·0	—	—	—	—
(94½)	—	286·0*	—	20·2	18·8	15·1*	0·94*
96	907	—	20·3	—	—	—	—
(103½)	—	41·0	—	21·1	19·7	2·1	0·68
111	1115	—	23·8	—	—	—	—
(115½)	—	23·0	—	21·5	20·1	1·1	0·53
120	1184	—	20·7	—	—	—	—
(130½)	—	2·9*	—	21·5	20·1	0·14*	0·01*
141	1204	—	21·5	—	—	—	—
(150)	—	35·7	—	21·5	20·1	1·7	0·64
159	1418	—	20·1	—	—	—	—
(160½)	—	—	—	21·5	20·1	(Means of 7 values)	
162	—	—	23·0	—	—	2·0	0·64

Subscript to Table II

Col. 1 gives the series of final time-values of the twelve experiments as in Table I, together with the mid-time point of each interval between these values, these latter within brackets. Col. 2 repeats from col. 5 of Table I the rising series of dry weight values for 100 embryos. In col. 3 appear the calculated rates of dry weight increase (G) over the intervals between the data recorded in col. 2. These values express gain of dry weight per 100 embryos per 3 hr. and are aligned with the mid-time points in brackets of col. 1. The respiration values in the succeeding columns of this table are all in hexose units. Those for the whole seedling (R_1+R_2) in col. 4 are derived from the CO_2 values in col. 2 of Table I by multiplying by the factor 0·68 ($C_6H_{12}O_6/6CO_2$). From these observed values of respiration at the ends of the individual experiments the values corresponding to the time points in brackets are obtained by interpolation. These interpolated values in col. 5 were derived by inspection of the continuous curve in fig. 8 based on the data in col. 4. Col. 6 contains the rates for the embryos only, obtained from the previous column by deducting R_1, the respiration rate for the endosperm, taken as 1·4 mg. hexose per 3 hr.

In col. 7 we have the direct ratio between growth and respiration, while in col. 8 we give to the ratio the form that has been called the "utilization coefficient". These are based on the values given for the concurrent G and R_2 values at each of the points of time in brackets. For cols. 7 and 8 the means of seven cases are given at the foot of the column, the values marked * having been discarded as anomalous.

From a study of the data of the two tables certain characteristics of the relations of growth and respiration become prominent.

For each index of growth in cols. 3, 4 and 5 of Table I it can be said that for the first 50 hr. the acceleration of growth rate is relatively slow; and that, after this first phase, marked accelerations of growth rate set in. This acceleration fades away about hour 90 into a third phase of practically constant growth rate, whichever index of growth we make use of. Only one of these indexes is plotted in graphic form in this paper, namely, the dry weight index as set out by curve *D.W.* in fig. 8. The points are rather

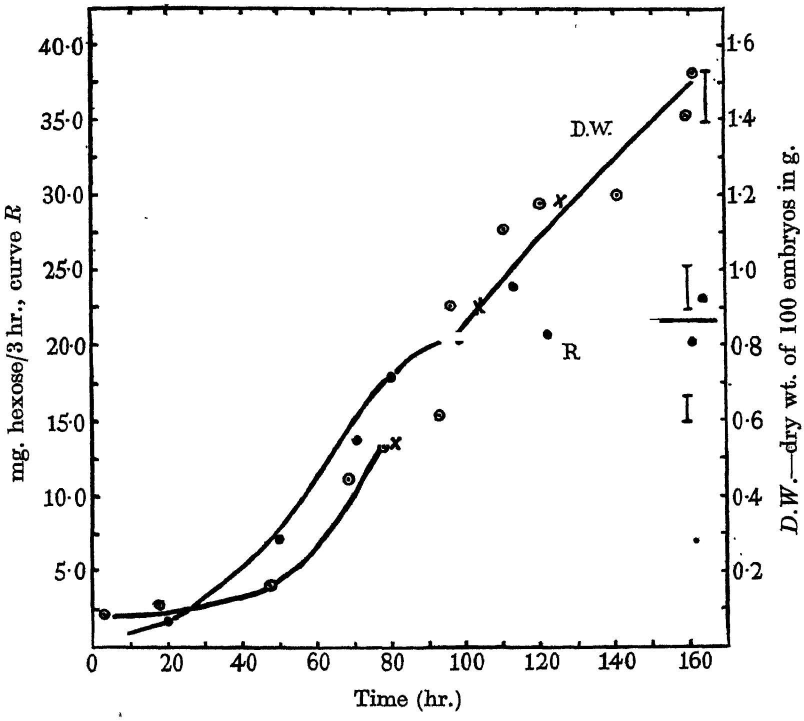

FIG. 8—The curve *D.W.* represents the rising course, with time, of the dry weights of a set of samples of 100 embryos of S.T.S. 1929 barley (data in dot circles from Table I, col. 5). The three crosses serve as a first approximation towards a smooth curve through the points (see text).

The three vertical lines to the right of the figure indicate the variation of duplicate weighings (i.e. difference between weights of two equivalent samples of 100 embryos estimated by weighing divided samples) when the embryos have attained three different stages of growth.

The curve *R* represents the drift of $R_1 + R_2$, giving the respiration rate, in hexose units, of whole S.T.S. 1929 barley seedlings, with time. Each point represents the seedling respiration rate during the 3 hr. immediately preceding the dissection of the embryos and the taking of growth measurements. The data are given in Table II, col. 4.

scattered so crosses have been introduced among the points, calculated as "centres of gravity" of successive groups of points. The straight line drawn through these centres after hour 90 indicates that we have here an approximation to a uniform growth of 0·03 g. dry weight gain/3 hr. at the temperature of 22° C. Finally there is some sign of deceleration of growth rate setting in.

A similar phenomenon dominates our observations of respiration rate. It will be recalled that at about hour 90 the respiration curves of figs. 3 and 4 turn over from rising rates to a state of oscillation about a constant mean upper limiting value. The serial set of respiration data in col. 2 of Table I shows the same effect, the last six readings varying about a mean value of 31·7 mg. CO_2. Fig. 8 presents a respiration curve to illustrate this feature, utilizing the data of col. 4 of Table II where the respiration is given in hexose units.

In cols. 7 and 8 of Table II we present a set of nine individual ratios between growth rate, G, and respiration rate, R_2. As the values of G and R_2 drawn upon had to be obtained from twelve separate germination experiments, a wide range of variation of ratios must be expected. In two regions of experimentation quite anomalous values were arrived at and these, marked with an asterisk, have been rejected, while the means of the other seven are set out at the foot of the table.

The ratios are presented in two forms. The direct ratio in col. 7 shows that the rate of deposition of hexose in growth of dry weight is about twice as fast as the rate of consumption of hexose in respiration. There is no sign of a directional drift among the observed empirical ratios throughout germination. In col. 8 the ratio is given the form of the "utilization coefficient",* being the ratio of achieved growth to total translocated stuff, T. Here $G/(G+R_2)$ indicates the proportion of hexose entering the embryo which is converted into new tissue (a negligible part of it being deposited as reserve carbohydrate) to the total hexose translocated.

The mean of the seven values of the "utilization coefficient" is here 0·64, with a standard error of 0·04. This particular value indicates a correlative value for G/R_2 of 1·78 which we shall use for future calculations though it is slightly lower than the direct value of 2·0 set out as the mean of seven selected values in col. 7. The state of affairs revealed is, then, that during the development of a barley plant from the embryo in the seed to a plant of 10 cm. or more in shoot length the embryo utilizes approximately 64 % of the hexose rendered available to it by translocation from the endosperm to form new tissues. Simultaneously approximately 36 % is consumed in respiration, which provides the energy required for the formation of these new tissues, for maintenance of the vital state of the protoplasm and for doing work against the external environment. There is, then, good evidence of a linkage between the processes of respiration and growth, which is maintained throughout the period of germination.

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* The term "utilization coefficient" was used by Terroine and others (1922, 1924) to describe the ratio of dry weight increase of a plant to the total uptake from (*a*) the surrounding medium in the case of a mycelium, or (*b*) the endosperm (measured by loss in dry weight) in the case of a germinating seed plant.

Calculations of the coefficient according to method (*b*) gave, with the present data, very variable values owing to inaccuracies in the endosperm dry weights due to loss of endosperm material during dissection. The method of calculation described in our text was therefore adopted.

Terroine, Bonnet and Joessel (1924) obtained similar results for their evaluation of the utilization coefficient in rice, sorghum, etc. They found no variation in the coefficient during the development of a seedling and also little or no difference between species of plants having seeds with endosperms of similar chemical composition. The value of the coefficient was determined by the chemical nature of the seed reserve; starchy seeds had relatively low coefficients, e.g. rice approximately 60 %, while oily seeds had relatively high coefficients, e.g. *Linum usitatissimum* approximately 90 %.

Magnitude of the Various Functions Co-operating in the Germinating System—It now becomes possible to present a graphic picture of the magnitudes of the rates of the contemporaneous carbohydrate events in the endosperm and embryo. Fig. 9 presents the drift of the essential values of the original schema set out on p. 321. The derivation of most of the values is given in the subscript to the figure. To this we may add that H—the carbohydrate hydrolysis in the endosperm—will be slightly greater than T by the addition of R_1, but this curve does not call for a separate line in the graph.

The magnitude of the reversible reserve deposit in the embryo, P (see p. 331), is held to be a negligible deduction from the values of G in this presentation.

The falling curve of respiration rate observed for embryos isolated from their endosperms is added to show the effect of sudden cessation of translocation supplies from the endosperm upon respiration in the embryo.

The dominant feature of the graph is the form of the rate of translocation, which is at first slow, then increases and finally exhibits an approximately constant rate maintained for at least several days before the experiments were broken off.

Respiration Rate per Unit Mass of Embryo Tissue—Fig. 10 shows, by curve *A*, the development of the respiration rate per gram dry weight of embryo tissue in the germinating seedling. Its derivation is explained in the subscript to the figure. Curve *A* cannot be regarded as more than a general trend suggested by the rather scattered data but it is possible to resolve its course into three phases:

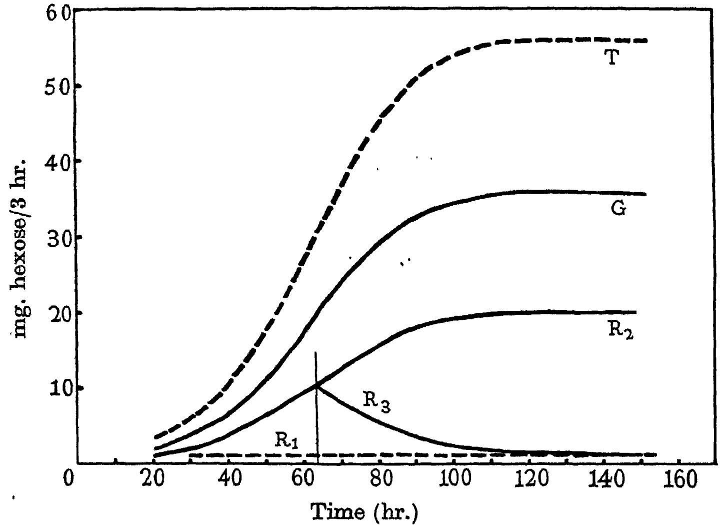

FIG. 9—Diagrammatic presentation of the correlated developmental changes of rate of the carbohydrate functions of the barley seedling, according to the schema given on p. 321.

Lettered curves are given for T, G, R_2 and R_1, all values being hexose or dry weight units per 3 hr.

The derivation of these values is as follows: From the observed rates of seedling respiration (R_1+R_2), R_2, the respiration of the embryo, is obtained by deduction of the constant value of 1·4 mg. hexose as allowance for the endosperm respiration, R_1. The G curve is drawn on the basis of $R_2 \times 1{\cdot}78$ (see p. 336) whence T becomes $R_2 \times 2{\cdot}78$.

For comparison with R_1 and R_2 there is added R_3 for the respiration of the isolated embryo (see fig. 6).

(1) From 0 to approximately 50 hr., a region of rapid acceleration of the respiration rate per unit mass of tissue.

(2) Approximately from 50 to 100 hr., a phase of markedly falling respiration rate.

(3) From 100 hr. onwards, a region of less rapidly falling respiration rate than that of phase (2).

The first phase of steeply rising respiration rate per unit mass suggests that at this stage activation of respiratory centres is taking place, probably without increase in number. Little increase in dry matter (i.e. formation of new tissue) occurs during this period, the respiration rate being, presumably, limited by the number of active respiratory centres.

During the second phase formation of new tissues is taking place more and more rapidly and the respiratory centres are being moved by extension of tissues farther from the endosperm, thus increasing the length of the diffusion path along which the endosperm hydrolysis products have to travel to them. The number of active centres of respiration is increasing while the respiration rate per unit dry matter falls considerably.

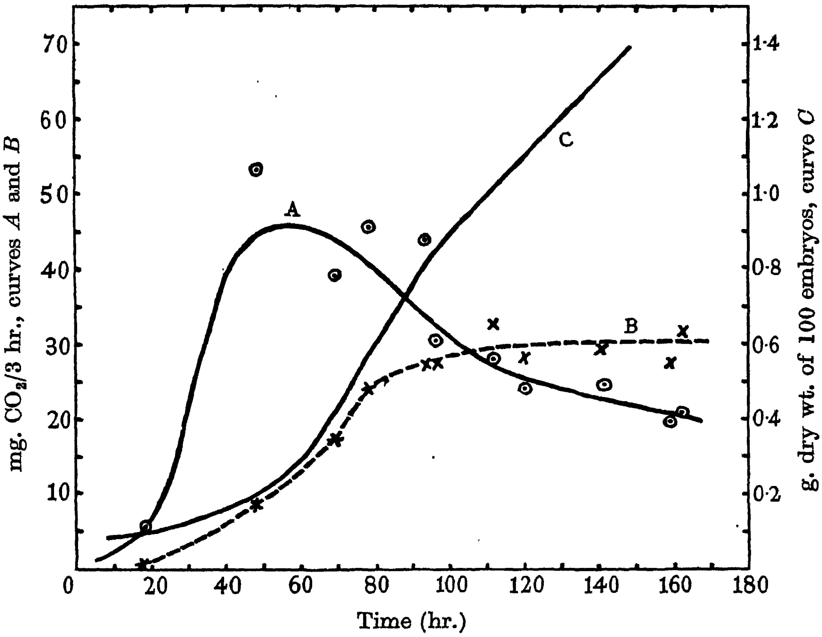

Fig. 10—The respiration rate of embryo tissue, per 1 g. dry weight (S.T.S. 1929), is shown at different periods of development by the circles through which a smooth curve A is arbitrarily drawn. These values are derived from the values plotted in the two other curves, B (CO$_2$ per 100 embryos) and C (dry weight of 100 embryos), by the ratio $A = B/C$. The numerical values of these three curves will be found in Table I: A in col. 8, B in col. 7 and C in col. 5. The form of B corresponds to the form of R in fig. 8, the respiration being there presented for the seedling in hexose units, but here for the embryo in CO$_2$ units. The dry weight smooth curve C is a replica of *D.W.* in fig. 8, but the values used in calculating A are the directly observed values recorded in Table I, col. 5.

In the third phase the respiration of the whole seedlings and the growth rate of the embryos is constant, both being limited by the translocation rate from the endosperms. This increasing addition to the dry matter of the embryo, in association with a constant rate of translocation, must result in a steady decrease in the respiration rate per unit mass of embryo tissue.

Balance Statement for the Sum of the Carbohydrate Metabolism of the Seedling System—It is of interest to check the total amounts of carbohydrates utilized by the embryos in growth and respiration with the loss in dry weight of their endosperms over an extended period.

The data for the period extending from hour 3 to hour 162 after the addition of water to the seeds provided by Table I may be presented in the form of a profit and loss account:

S.T.S. 1929 BARLEY FROM HOURS 3 TO 162

Embryo

	g.
Dry weight gain of 100 embryos during the 159 hr. period = (1·523–0·087)	=1·44
Loss of hexose due to respiration of embryos over same period = (0·75–0·07)*	=0·68
Total hexose accounted for by the embryo	=2·12

Endosperm

Total loss in dry weight over same period = (3·982–1·430)	=2·55
Loss due to endosperm respiration over same period	=0·07
Total hexose provided for translocation	=2·48

On these experimental data, the endosperm is credited with producing 0·36 g. more hexose than the embryo accounts for. We should regard this loss on the balance sheet as explained by the almost liquid state of the endosperm residues at hour 162 so that they cannot be wholly dissected off the embryo for estimation of dry weight.

We may also attempt an estimate of the proportion of the reserve carbohydrate in barley grains which is accounted for by the metabolism of these embryos up to hour 162 of their development.

Adopting the figures† 69·5 and 12·3 respectively as the percentages of carbohydrates and of water in "air-dry" barley grains, then if we assume that oven-drying at 100° C. drives off all the water content the percentage of carbohydrate in the dried seeds is 79·3. Taking, as the weight of 100 grains, the rather low value given by (weight of 100 endosperms + weight of 100 embryos) after 3 hr., i.e. 3·982 + 0·087 = 4·069 g. (see Table I), there is present $4{\cdot}069 \times \frac{79{\cdot}3}{100} = 3{\cdot}22$ g. of carbohydrate, all, presumably, available for growth and respiration. At the end of a further 159 hr. the endosperms have lost 2·55 g. and, assuming this to be all carbohydrate, only $(3{\cdot}22 - 2{\cdot}55) = 0{\cdot}67$ g. of carbohydrate remains in the endosperms. So, after 162 hr. at 22° C., when the seedlings have attained a shoot length of 11 cm., they have utilized somewhere about four-fifths of the carbohydrate reserve in the endosperm, and later will have only the residue to draw upon.

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* 0·75 = integration by summing squares of respiration curve of whole seedlings in fig. 8. 0·07 = total weight of hexose respired by endosperms in 159 hr. at the rate of 1·4 mg. hexose per 3 hr..

† The following percentage composition for barley grains is given in "The Technology of Bread-making" by Jāgo and Jāgo (1911): fat 1·9, carbohydrates 69·5, cellulose 3·8, proteins 10·1, mineral matter 2·4, water 12·3.

This paper covers part of the research work carried out in the Cambridge Botany School under the direction of Dr. F. F. Blackman. My thanks are due to him and to Mr. G. E. Briggs for advice and constructive criticism in the presentation of these results.

SUMMARY

The major features of the metabolic activity of young barley seedlings are: (1) the hydrolysis of starch in the endosperm, (2) the formation of new tissues in the embryo by growth in dry weight, and (3) the respiration of carbohydrates; which last can be studied (*a*) for the whole seedling, (*b*) for the detached embryo, and (*c*) for the endosperm alone after sterile dissection.

The linkage of these functions through translocation has been formally set out on p. 321 and the parallel development of them has been studied quantitatively in carbohydrate units through the first 162 hr. of germination of Spratt-Archer barley in a current of air in an experimental chamber at 22° C. (see fig. 9).

Respiration—The time course of the respiration rate for whole seedlings shows a sigmoid form in its rise to a well-defined upper limiting value, set by the amount of carbohydrate being translocated from the endosperm. This limited phase is reached at about hour 90 and it has been followed till about hour 160, showing only moderate fluctuations round the value of 32 mg. CO_2/3 hr./100 seedlings. Beyond hour 160, where about four-fifths of the endosperm has been exhausted, the functions have not been followed into their decline.

Some sterile dissections of the endosperms from the embryo have been achieved and the vitality of the isolated endosperm demonstrated by its

continuous respiration—at the low rate of about 2 mg. CO_2/100 endosperms.

The respiration of the isolated embryos has also been followed in a set of cases. A rapid fall of respiration sets in immediately and the rate declines in a geometric progression to a low starvation value of about 1·8 mg. CO_2. The features indicate that only a very small stock of available reserves has been deposited in the embryo at the early stage.

Growth—Our survey requires an index of growth rate in terms of dry weight increase. For the construction of a curve of increasing dry weight through the first 160 days of germination twelve sets of seedlings were started and broken off at suitably distributed points of time for determination of the dry weights attained by the embryo. The data show a considerable scatter and the precise form of the smooth curve compounded from them in fig. 8 is not very well established. The slopes of the curve show the usual initial course of the acceleration of growth-rate up to hour 90, while after that the rate proceeds fairly uniformly for the remaining 70 hr. of experimentation.

From the numerical dry weight data a sequence of growth-rate values (G) was calculated throughout the period of 160 hr., in order that utilization of carbohydrate in growth might be compared with the consumption of carbohydrate in respiration, R_2, over successive periods of development.

Relation of Rates between Concurrent Respiration and Growth—Nine pairs of growth and respiration values were obtained in this way and these are set out in Table II in the forms G/R_2 and $(G/G+R_2)$, the latter being the form known as the "utilization coefficient", viewing growth as the utilized fraction of the total carbohydrate supply. Excluding two very aberrant magnitudes, the remaining seven cases show no sign of any drift in the growth/respiration relation. We may therefore put forward, as a first approximation, the mean values that they provide; namely $G/R_2 = 1{\cdot}78$; with G and R_2 as accounting for, respectively, 64 and 36 % of the total available carbohydrate supply entering the embryo.

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616—006.46: 547.68

The Production of Cancer by Pure Hydrocarbons—IV

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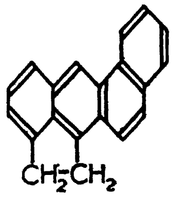
[Plates 14, 15]

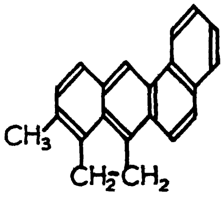
Tests for carcinogenic activity on about 140 compounds were recorded in earlier parts of this series (Part I, Cook, Hieger, Kennaway and Mayneord 1932; Part II, Cook 1932; Part III, Barry, Cook, Haslewood, Hewett, Hieger and Kennaway 1935). These showed that the most active compounds are methylcholanthrene and 3:4-benzpyrene, in the sense that these compounds gave tumours which appeared more rapidly than in the case of any other compound. The carcinogenic activity of these two compounds is also of interest on account of the facts that methylcholanthrene is obtained by chemical means from the naturally occurring bile acids, cholic and deoxycholic acids, and 3:4-benzpyrene is present in coal tar. Experiments now recorded have shown that a similar high order of carcinogenic activity is likewise shown by certain other substances containing the same ring systems as these two compounds, although the activity may be readily destroyed by slight modification of molecular structure. We have also extended our general studies of the relationship between molecular structure and carcinogenic activity among compounds related to 1:2-benzanthracene. The technique has been described already, and no further details need be given. Except where otherwise stated the compounds were applied twice weekly in 0·3 % solution in benzene to the skin of mice.

CHOLANTHRENE DERIVATIVES

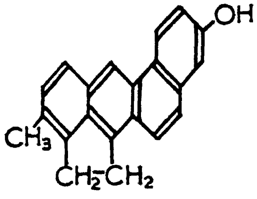
The parent hydrocarbon of this group, cholanthrene, has been prepared synthetically by several different methods, and it is evident from the results summarized in Table I and fig. 1 that cholanthrene is no less potent than methylcholanthrene in producing malignant tumours of the skin of mice, when applied in dilute solution in benzene. A similar conclusion concerning the relative potencies of these two compounds was arrived at by Shear (1936), who studied the production of sarcomas by inserting pellets of cholesterol containing the hydrocarbon into the subcutaneous tissue of mice. In the light of our previous results with homologues of 1:2-benzanthracene it would seem likely that the carcinogenic activity of methylcholanthrene is determined mainly by the fact that this hydrocarbon is a 5:6-substituted derivative of 1:2-benzanthracene. From this standpoint it is surprising that cholanthrene should be so highly active, for this hydrocarbon lacks the substituent at position 6 of the benzanthracene ring system, and it has been found among the simple homologues of benzanthracene that the 5-monosubstituted derivatives, though carcinogenic (see p. 355), are less effective than the 5:6-disubstituted derivatives (Part III). Thus, the second point of attachment of the five-membered ring of cholanthrene, at position 10 of the benzanthracene ring system, appears to reinforce the influence of substitution at position 5 to a greater extent than is the case with a substituent at position 6. On this view it might be expected that activity would be shown by 10-monosubstituted benzanthracenes. The only such compounds hitherto examined are 10-*iso*propyl- and 10-benzyl-1:2-benzanthracenes, which were completely inactive. In our experiments with our recently synthesized 10-methyl-1:2-benzanthracene, which unfortunately had to be abandoned at an early stage on account of an epidemic among the mice, we have been anticipated by Fieser and his collaborators (1936, 1937), who report that both 10-methyl-1:2-benzanthracene and 5:10-dimethyl-1:2-benzanthracene show the high degree of carcinogenic activity characteristic of cholanthrene and methylcholanthrene. Therefore, substitution at 5:10 appears to be more effective in promoting carcinogenic potency than substitution at positions 5:6, irrespective of whether or no these substituents form part of a new ring.



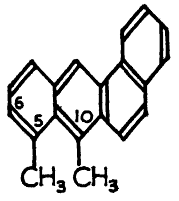
Cholanthrene



Methylcholanthrene



Methylhydroxycholanthrene



5:10-Dimethyl-1:2-benzanthracene.

It was suggested in Part III that the comparative facility with which methylcholanthrene may be prepared chemically from deoxycholic acid leads to the possibility that abnormal metabolism of the bile acids may result in the production *in vivo* of methylcholanthrene or a related compound. As most of the naturally occurring compounds of the sterol group have the characteristic hydroxyl group at position 3 (sterol system of numbering), it seems probable that this hydroxyl group would be retained if such a series of reactions as that envisaged actually occurred in the body. In this way deoxycholic acid would be converted, not into methylcholanthrene, but into the methylhydroxycholanthrene of the structure shown. This compound has been synthesized and has been applied to the skin of mice for 10 months and its methyl ether for 12 months without producing tumours (Table I). Thus it is clear that the introduction of oxygen into this position of the methylcholanthrene molecule has a powerful adverse influence on the carcinogenic activity. In order to determine whether this influence is due to the nature of the substituent or to the position of substitution it will be necessary to synthesize and test new derivatives of cholanthrene, and such experiments are in progress. Negative results were also obtained, in an experiment lasting for more than 2 years, with dehydronorcholene, the immediate precursor of methylcholanthrene, of which it is an almost completely hydrogenated derivative. Negative results were likewise obtained with a synthetic compound of

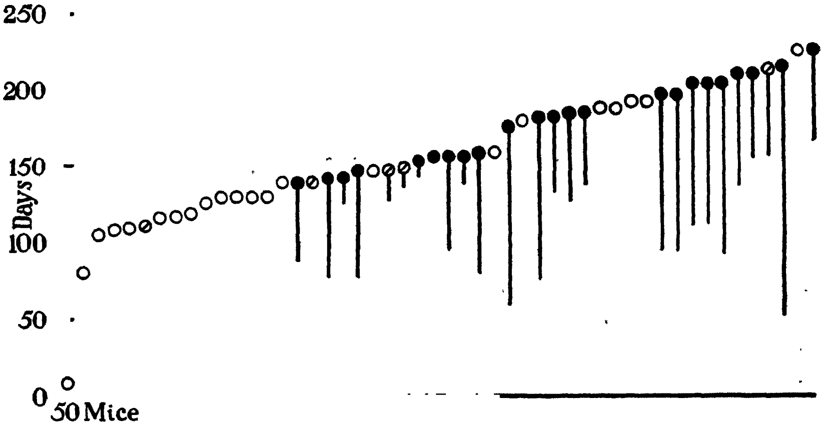
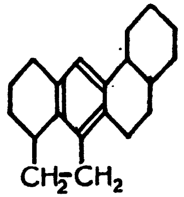
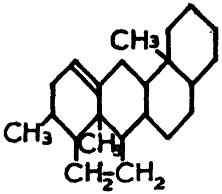
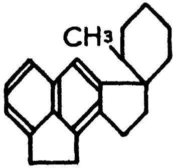
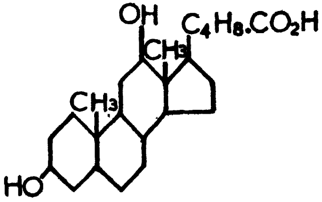


FIG. 1—Cholanthrene. In this and figs. 2–8 the centre of each circle marks the day of death of one mouse, reckoned from the beginning of the experiment. ● = epithelioma; ⊘ = papilloma; ○ = no tumour. The vertical lines represent the period of growth of a tumour, from the day when it was first observed to the death of the mouse.

TABLE I—CHOLANTHRENE AND RELATED COMPOUNDS

Compound	Method of preparation	Animals					Tumours	
			Initial no.	Alive after		Day of death of last animal	Epi-thelioma	Papil-loma
				6 months	1 year			
Methylcholanthrene (0·1 % in benzene)	Cook and Haslewood (1934)	Stock mice	20	13	0	238	3	2
Methylcholanthrene (0·3 % in benzene)	Cook and Haslewood (1934)	,,	10	7	0	223	5	0
Methylcholanthrene (0·3 % in benzene)	Cook and Haslewood (1934)	Simpson mice	20	0	0	141	0	2
Methylcholanthrene (0·3 % in benzene)	Cook and Haslewood (1934)	C.B.A. mice	18	14	0	219	15	2
Methylcholanthrene (0·3 % in benzene)	Cook and Haslewood (1934)	Rats	22	12	4	657	?2	0
Cholanthrene	Cook and Haslewood (1935 *b*)	Stock mice	50	17	0	224	23	5
Methylhydroxycholanthrene (0·3 % in acetone)	Cook and de Worms (unpublished)	,,	10	6		3 alive day 322*	—	—
Methylmethoxycholanthrene	Cook and de Worms (unpublished)	,,	10	5	2	2 alive day 370*	—	—
Dodecahydrocholanthrene	Cook and Haslewood (1935 *a*)	,,	20	16	6	618	0	0



Dehydronorcholene 	Wieland and Schlichting (1925)	Stock mice	40	22	10	740	0	0
1:2-*cyclo*Pentenoacenaphthene-3′-*spirocyclo*-2″-methyl*cyclo*hexane 	Cook, Haslewood and Robinson (1935)	,,	20	16	10	743	0	0
Deoxycholic acid 		,,	20	17	8	776	1	0
			6 ♀ (castrated)	5	4	476	0	0

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* 5 March 1937.

a very similar nature, dodecahydrocholanthrene. In fact, the available evidence seems to show that among the polycyclic hydrocarbons a completely aromatic ring system is necessary for the manifestation of carcinogenic activity.

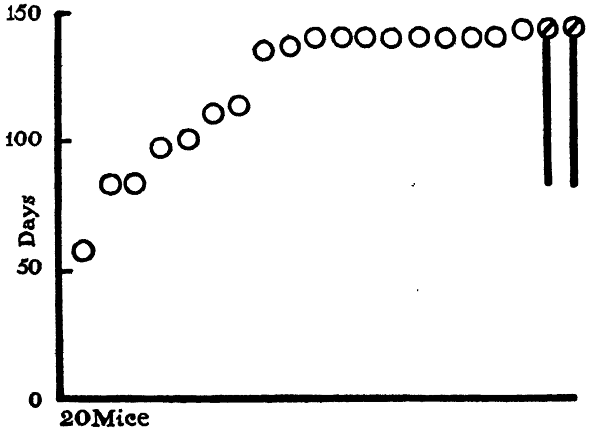

FIG. 2—Methylcholanthrene (Simpson mice).

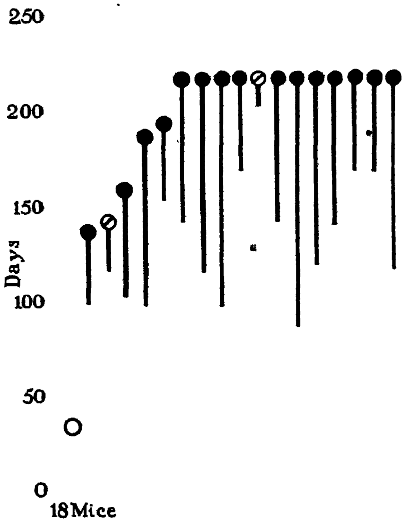

FIG. 3—Methylcholanthrene (C.B.A. mice).

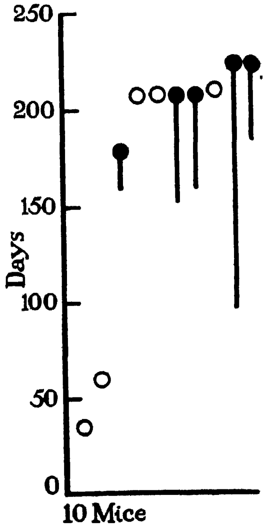

FIG. 4—Methylcholanthrene (stock mice).

In tests for carcinogenic activity carried out in this Institute the animals used have almost invariably been of mixed stock as obtained from the dealer. In Table I and figs. 2, 3 and 4 are recorded the results of application of methylcholanthrene to the skin of three series of mice, two of which were of pure strain. It is evident from comparison of the results obtained with C.B.A. mice, in which spontaneous mammary cancer is hardly ever observed, with those obtained with ordinary stock mice, that the C.B.A. strain has no inherent power of resistance to the carcinogenic action of methylcholanthrene. On the other hand, only two papillomas were obtained in twenty mice of the Simpson strain, which show a high incidence of mammary cancer in the females. The mice used in this experiment had all died by the 141st day, so that the results are not strictly comparable with those obtained with the other two series, which lived appreciably longer. Nevertheless, it seems evident from figs. 2, 3 and 4, and from fig. 7 of Part III, that the Simpson mice did not respond normally to the carcinogenic action of methylcholanthrene. The small number of mice used precludes any very wide generalization from these results, which are, however, in harmony with those of Kreyberg (1925) who bred out two strains of mice, one of which gave a high incidence of mammary cancer, but were resistant to painting with coal tar, while the other showed a low incidence of mammary cancer but readily yielded epitheliomas with coal tar.

In an experiment lasting for more than 2 years, one epithelioma was obtained in a series of mice treated with deoxycholic acid, the naturally occurring precursor of methylcholanthrene. The experiment is being repeated with larger numbers of mice, and until further tumours are forthcoming it is probably wiser to regard this one tumour as a rare example of a "spontaneous" epithelioma than to attribute a weak carcinogenic action to this bile acid.

EPITHELIAL TUMOURS OF THE SKIN PRODUCED IN RATS BY METHYLCHOLANTHRENE

On account of the high carcinogenic activity towards the skin of mice, and towards the connective tissue of both rats and mice, it seemed of interest to apply this hydrocarbon to the skin of rats, which, as is well known, rarely responds to the carcinogenic action of coal tar. Table I shows that tumours of the skin were produced by methylcholanthrene in some rats which lived sufficiently long. Methylcholanthrene (0·3 % in benzene) was applied twice weekly to the interscapular skin of twenty-two rats (Wistar strain); the substance appeared to be toxic to them, as only four rats lived for more than 1 year. Papillomas appeared in two rats (on the 178th and 329th day) on the area painted; these tumours grew very slowly until death (on the 461st and 657th days respectively). The microscopic appearances were peculiar; one tumour consisted of deeply staining spindle-shaped cells somewhat resembling those of a rodent ulcer in man (fig. 9, Plate 14) and showing in parts an alveolar appearance (fig. 10, Plate 14); the other consisted of similar tissue in some portions, and elsewhere of squamous epithelioma of the ordinary type (fig. 11, Plate 15), though this latter shows a columnar arrangement of the cells in places (fig. 12, Plate 15). No definite penetration of muscle and no metastases were found, hence these tumours are classed as "?Epithelioma" in Table I. This appears to be the first instance in which tumours of the skin have been produced in rats with a pure compound.*

BENZPYRENES

Whether the carcinogenic activity of the coal-tar hydrocarbon, 3:4-benzpyrene,† is a special attribute of this particular pentacyclic system, or whether it is fundamentally due to the 1:2-benzanthracene ring system which the compound contains cannot yet be assessed, as no simple benzanthracene derivatives with substituents at positions 9 or 1′ have yet received adequate test, although as a preliminary step in this direction we have in progress an experiment with 9-methyl-1:2-benzanthracene. It is at any rate certain that 3:4-benzpyrene is not an unique example of a carcinogenic hydrocarbon containing this ring system, for preliminary tests have shown that 1:2:3:4-dibenzpyrene, 7-methyl-1:2:3:4-dibenzpyrene and 3:4:8:9-dibenzpyrene are carcinogenic agents of considerable potency.‡ Table II shows that epitheliomas were obtained in five of six mice to which these three hydrocarbons were applied, and the early appearance of these tumours is an indication of the high order of activity. This is especially of interest, for 3:4:8:9-dibenzpyrene is

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* It is perhaps just worth remark, though one would wish to avoid any speculation, that these tumours of low malignancy, in a resistant species, resemble in appearance the rodent ulcer of man, which latter tumour is also of low malignancy. Hence the occurrence of this histological type in the resistant species (rat) suggests that the comparatively non-malignant nature of the rodent ulcer in man may be due, not as is perhaps generally assumed, to a character inherent in the tumour itself, but to a capacity for resistance in the particular person affected.

† This was originally termed 1:2-benzpyrene, in conformity with the systematic numbering of the pyrene ring system proposed by Patterson (1925). In order to avoid perpetuating two separate systems of numbering we are reverting, at the request of the Editor of Beilstein's "Handbuch der organischen Chemie", to the pyrene numbering used in the older literature, whereby the carcinogenic hydrocarbon isolated from coal tar is denoted as 3:4-benzpyrene.

‡ We are indebted to Dr. E. Clar, who synthesized these three hydrocarbons, for generously placing specimens of them at our disposal.

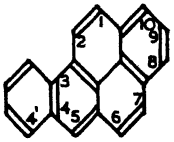
TABLE II—DERIVATIVES OF 3:4-BENZPYRENE

		Mice				Tumours	
			Alive after				
Compound	Method of preparation	Initial no.	6 months	1 year	Day of death of last mouse	Epi- thelioma	Papil- loma
1:2:3:4-Dibenzpyrene	Clar (unpublished)	2	1	0	262	1	0
7-Methyl-1:2:3:4-dibenzpyrene	Clar (unpublished)	2	2	0	284	2	0
3:4:8:9-Dibenzpyrene	Clar (unpublished)	2	2	0	310	2	0
4′-Hydroxy-3:4-benzpyrene (0·3 % in acetone)	Fieser, Hershberg and Newman (1935)	10	10	2	2 alive, day 395*	—	—
4′-Methoxy-3:4-benzpyrene	Fieser, Hershberg and Newman (1935)	10	5		2 alive, day 362*	—	—
3:4-Benzpyrene (0·3 % in acetone)	—	20			9 alive, day 122*	—	2 in living mice

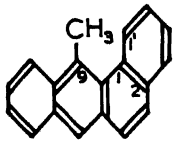
* 5 April 1937.

considerably less soluble than the highly active compounds which we had previously encountered, and the results provide evidence that the high activity of cholanthrene and 3:4-benzpyrene is not primarily due to their relatively high solubility, and also show that a very low solubility does not necessarily preclude the production of tumours by compounds, the molecules of which are endowed intrinsically with carcinogenic activity. An added significance thus attaches to the negative results obtained with certain complex hydrocarbons of very low solubility, such as picene, 2′:3′-naphtha-1:2-phenanthrene, 2′:3′-naphtha-2:3-phenanthrene (Part I); 2′:3′-phenanthra-1:2-anthracene, and 4:5-benz-10:11-(1′:2′-naphtha) chrysene (Part II). A more extensive test, with a larger number of mice, is in progress with 3:4:8:9-dibenzpyrene, and another series of mice is being treated with anthanthrene, a hydrocarbon of similar structure, from which is derived the important vat dyestuff, anthanthrone. Since this latter experiment was started we have found that Domagk (1936) has reported negative results with anthanthrene.

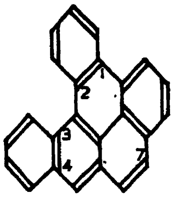
The effect of the hydroxyl group in suppressing carcinogenic activity, to which allusion has been made in connexion with methylcholanthrene, has also been observed with the 3:4-benzpyrene molecule; negative results have been obtained in experiments not yet complete (Table II) with 4′-hydroxy-3:4-benzpyrene and its methyl ether.



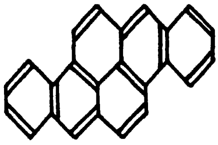
3:4-Benzpyrene



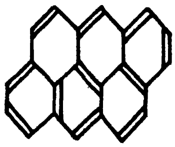
9-Methyl-1:2-benzanthracene



1:2:3:4-Dibenzpyrene



3:4:8:9-Dibenzpyrene

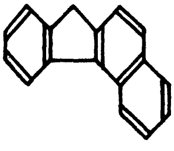


Anthanthrene.

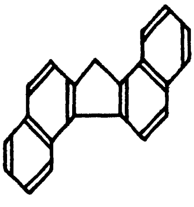
On account of the low solubility in benzene of methylhydroxycholanthrene and hydroxybenzpyrene, acetone has been used as the solvent in tests carried out with these two compounds. As this solvent has not hitherto been used in our tests a control experiment with acetone solutions of 3:4-benzpyrene is in progress. After 122 days tumours have been produced in two of nine mice still living, so that the use of acetone as a solvent appears not to prevent the substance from exerting its carcinogenic action.

FLUORENE DERIVATIVES

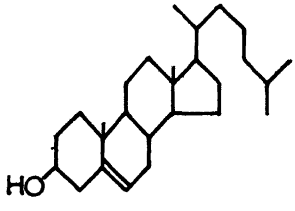
Most of the potent carcinogenic compounds contain the anthracene and the phenanthrene ring systems, and it seemed desirable to ascertain to what extent this is necessary for activity among the polycyclic compounds. In Part III we showed that certain acridine derivatives having formal resemblance to carcinogenic hydrocarbons were also active, and we have now extended these experiments to compounds containing the fluorene ring system. There are two isomeric benzfluorenes corresponding with 1:2-benzanthracene. Negative results with one of these (1:2-benzfluorene = chrysofluorene) have already been recorded (Part III), and we have now applied 3:4-benzfluorene to the skin of ten mice for nearly 1½ years without obtaining tumours (Table III). Carcinogenic properties were shown, however, by 1:2:5:6-dibenzfluorene in which a five-membered ring takes the place of the central aromatic ring of the carcinogenic 1:2:5:6-dibenzanthracene. Thus, the molecule of 1:2:5:6-dibenzanthracene may be modified by



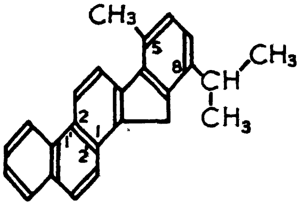
3:4-Benzfluorene



1:2:5:6-Dibenzfluorene

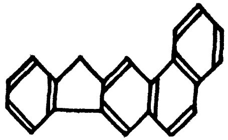


Cholesterol



5-Methyl-8-*iso*propyl-2′:1′-naphtha-1:2-fluorene.

TABLE III—COMPOUNDS RELATED TO FLUORENE

		Mice				Tumours	
			Alive after				
Compound	Method of preparation	Initial no.	6 months	1 year	Day of death of last mouse	Epi-thelioma	Papil-loma
3:4-Benzfluorene	Cook, Dansi, Hewett, Iball, Mayneord and Roe (1935)	20	13	6	537	0	0
1:2:5:6-Dibenzfluorene (pure specimen)	Cook, Dansi, Hewett, Iball, Mayneord and Roe (1935)	20	10	0	354	3	1
1:2:5:6-Dibenzfluorene not completely purified	Cook, Dansi, Hewett, Iball, Mayneord and Roe (1935)	10♂ 10♀	6 8	3 0	472 364	2 1	1 0
1′:2′-Naphtha-2:3-fluorene	Cook, Dansi, Hewett, Iball, Mayneord and Roe (1935)	5	4	4	1 alive, day 566*	—	—
							
5-Methyl-8-*iso*propyl-2′:1′-naphtha-1:2-fluorene	Cook, Hewett, Mayneord and Roe (1934)	20	9	6	636	0	0
8-Methyl-2′:1′-*naphtha*-1:2-fluorene	Bachmann, Cook, Hewett and Iball (1936)	20	18	10	582	0	0

* 5 March 1937.

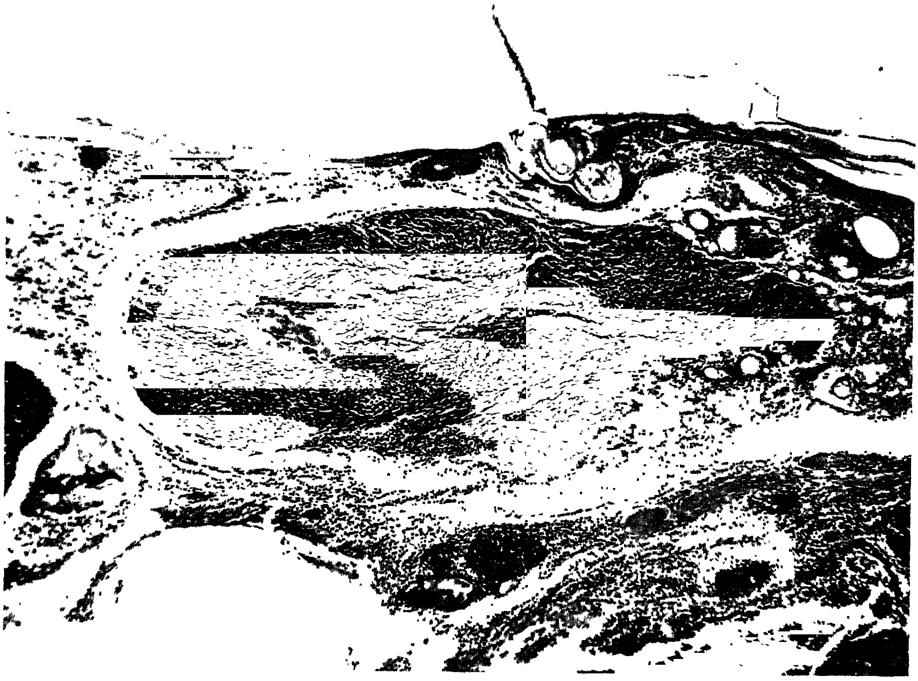


Fig. 9

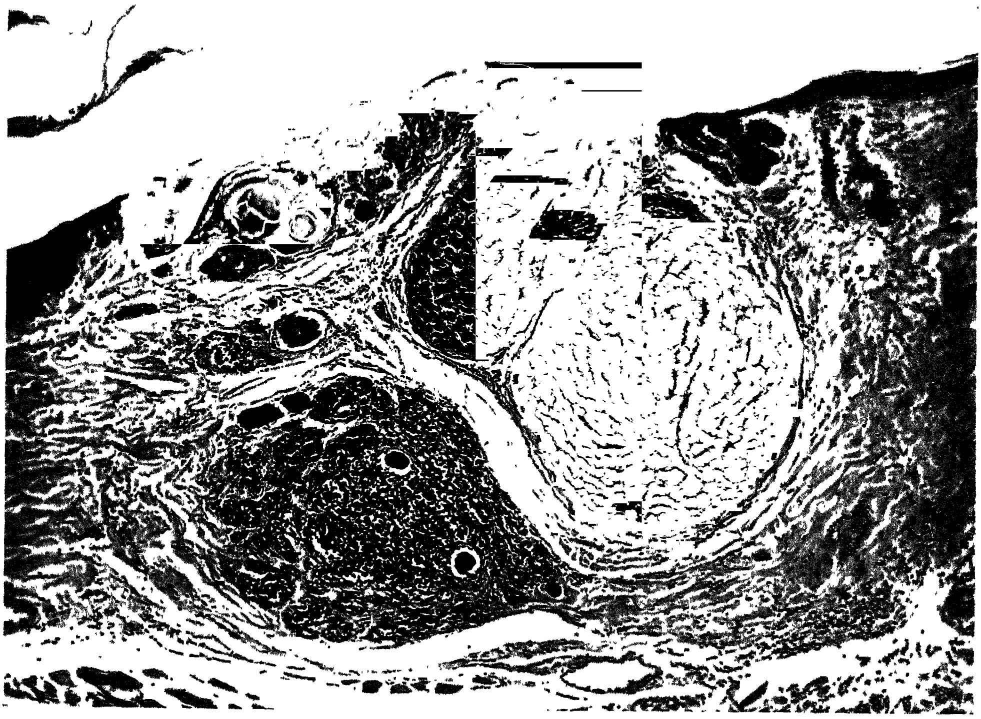


Fig. 10

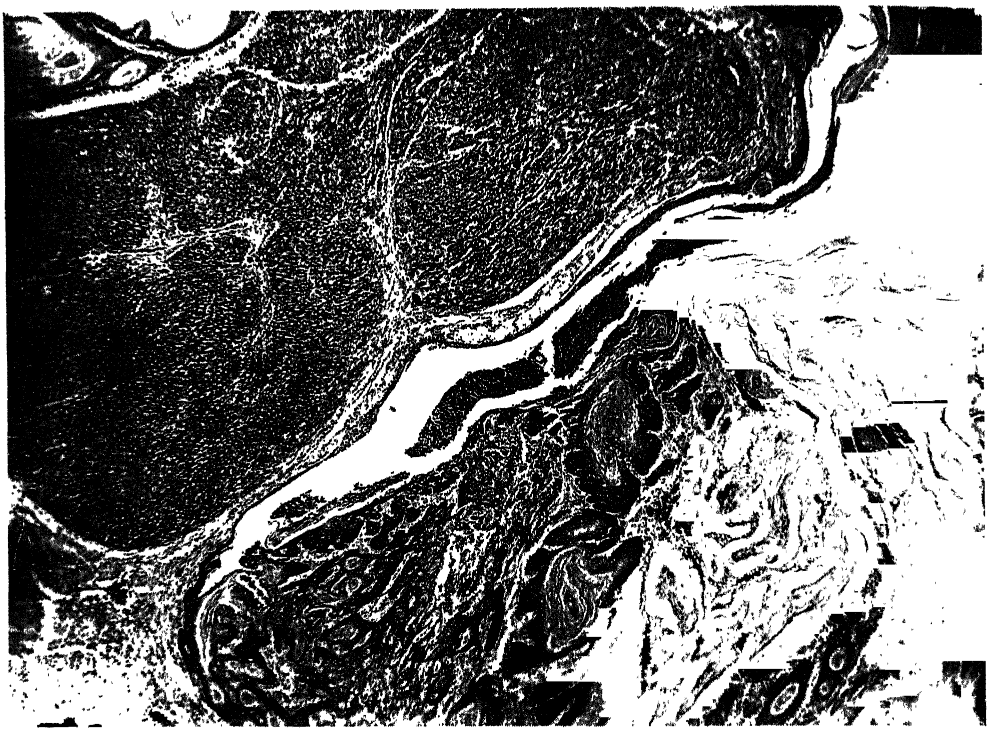


Fig. 11

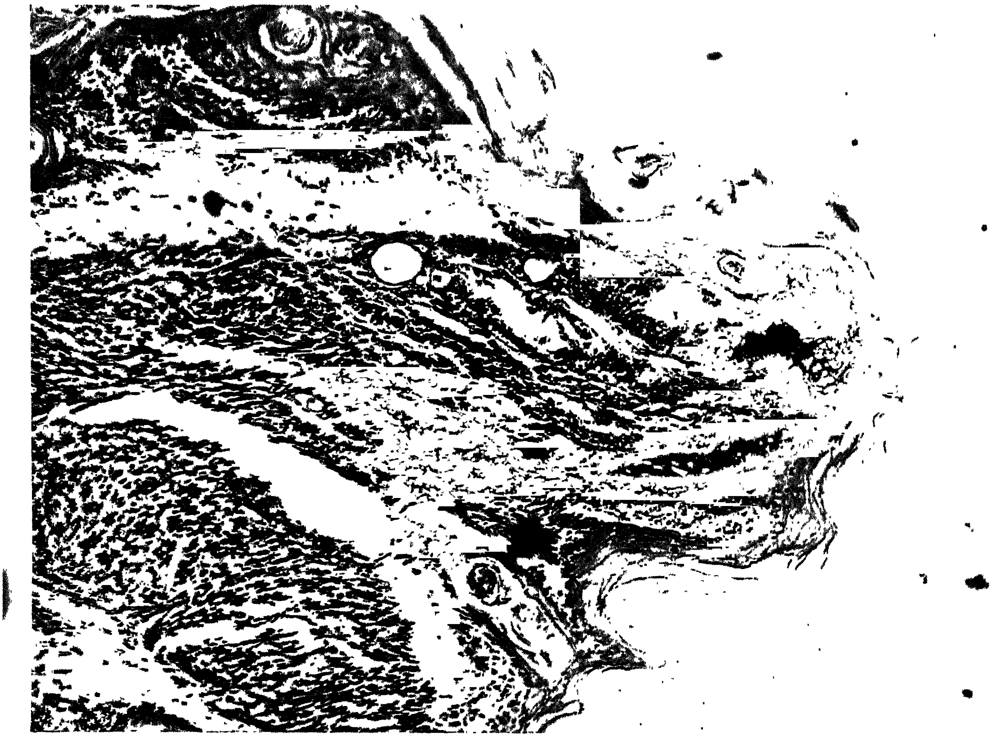
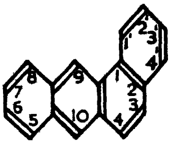


Fig. 12

introduction of nitrogen into the ring system *or* contraction of the central ring without complete loss of carcinogenic activity. Boyland and Brues (1937) have further shown that activity may be retained when *both* these changes are made, weak cancer-producing activity being shown by 1:2:5:6-dibenzcarbazole.

In view of the high carcinogenic activity of methylcholanthrene, which is an aromatic pentacyclic hydrocarbon derived from the bile acids, it seemed of interest to examine 5-methyl-8-*iso*propyl-2′:1′-naphtha-1:2-fluorene, which can be seen from the formulae to be derived from cholesterol by cyclization and dehydrogenation. A synthetic specimen of this hydrocarbon has given no tumours in an experiment lasting for nearly 2 years. Negative results have also been obtained (Table III) with two similar complex fluorene derivatives, 8-methyl-2′:1′-naphtha-1:2-fluorene and 1′:2′-naphtha-2:3-fluorene.

BENZANTHRACENE HOMOLOGUES



1:2-Benzanthracene.

Of the monomethyl derivatives hitherto reported, only the 5-methyl compound showed pronounced carcinogenic activity. Tests have now been made with the 5-ethyl and 5-*n*-propyl compounds, both of which proved active (Table IV, figs. 6 and 7). The former gave seven tumours (five epitheliomas and two papillomas) in a series of twenty mice, and the latter gave six tumours (three epitheliomas and three papillomas) in twenty mice. On this basis the two new homologues appear less active than 5-methyl-1:2-benzanthracene (fig. 5), which gave seven tumours (five epitheliomas and two papillomas) in ten mice, but the number of animals is insufficient to warrant any final conclusion on this point. On the other hand, tumours appeared earlier, on the whole, with the two new compounds. The results indicate that substitution in position 5 is especially favourable for carcinogenic activity among 1:2-benzanthracene derivatives, and we are engaged in a study of the higher homologues. Tests are also in progress with three new monomethyl derivatives of 1:2-benzanthracene (with substituents at positions 9, 10 and 4′), bringing the total number of methylbenzanthracenes

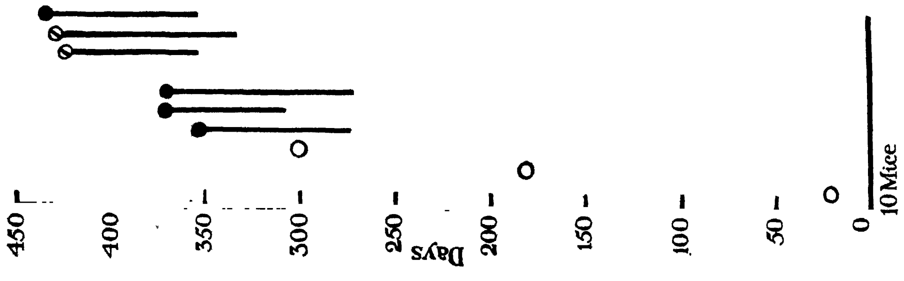


Fig. 5—5-Methyl-1:2-benzanthracene.

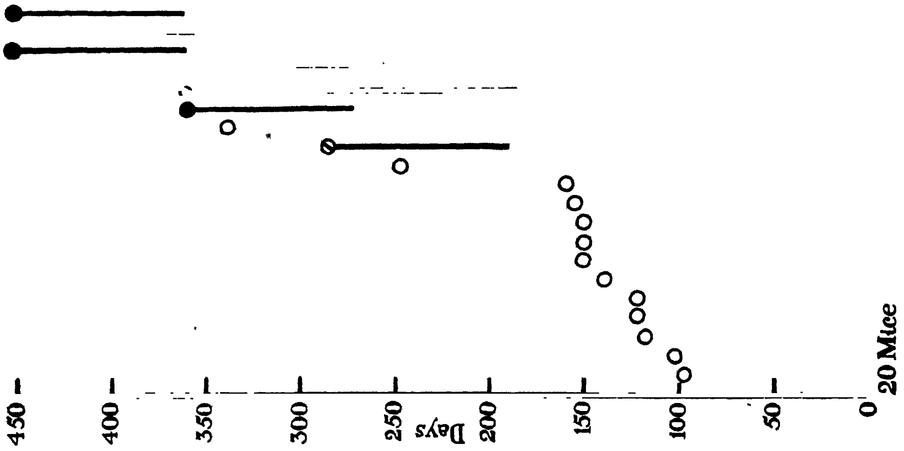


Fig. 6—5-Ethyl-1:2-benzanthracene.

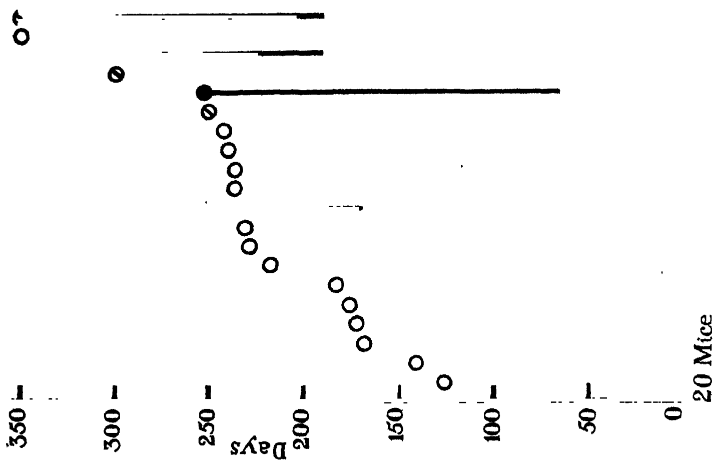
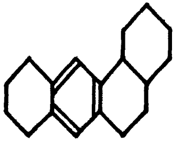
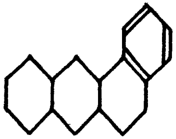


Fig. 7—5-*n*-Propyl-1:2-benzanthracene.

Table IV—Derivatives of 1:2-Benzanthracene and 3:4 Benzphenanthrene

Compound	Method of preparation	Mice				Tumours	
		Initial no.	Alive after		Day of death of last mouse	Epi-thelioma	Papil-loma
			6 months	1 year			
5-Ethyl-1:2-benzanthracene	Cook, Robinson and Goulden (1937)	20	9	3	455	5	2
5-*n*-Propyl-1:2-benzanthracene	Cook and Haslewood (1935 *a*)	20	15	0	347	3	3
Dodecahydro-1:2-benzanthracene, m.p. 87–88° 	Cook and Haslewood (1935 *a*)	20	16	2	654	0	0
Dodecahydro-1:2-benzanthracene, m.p. 71–72° 	Cook and Hewett (1934)	10	9	7	671	0	1
3:4-Benzphenanthrene	Hewett (1936)	10	4	2	2 alive, day 380*	—	—
		10	5		4 alive, day 267*	—	—
		20	12	5	4 alive, day 422*	—	—
2-Methyl-3:4-benzphenanthrene	Hewett (1936)	20	16	0	300	7	5

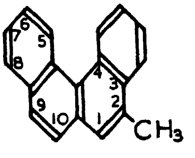
* 5 March 1937

now examined to ten, out of a possible twelve. No results can yet be reported except that 10-methyl-1:2-benzanthracene was applied to the skin of twenty mice, of which only four were alive on the 130th day. Of these, one died bearing a papilloma and one killed on the 260th day bore an epithelioma. These data do not suggest any high degree of carcinogenic activity, although Fieser and Hershberg (1937) report that subcutaneous injection into mice of 10-methyl-1:2-benzanthracene gave sarcomas which made their first appearance in 2 months, a degree of activity comparable with that of the cholanthrene group.

Tests have also been made with two isomeric dodecahydro derivatives of 1:2-benzanthracene, having melting-points of 71–72° and 87–88° respectively. In the case of the former compound one papilloma was obtained in a series of ten mice (Table IV).

3:4-BENZPHENANTHRENE

The observation (Part III) that 3:4-benzphenanthrene gave tumours, after a prolonged latent period, in a large proportion of the mice treated required further investigation, as although considerable precautions had been taken to ensure the use of a pure specimen of the hydrocarbon, such a result might be due to the presence of a small amount of persistent impurity of high carcinogenic potency. 3:4-Benzphenanthrene has now been synthesized by a completely different method from that used for the original specimen, and although no tumours have yet arisen in the mice to which the new sample has been applied (Table IV), the inherent carcinogenic activity of the ring system of 3:4-benzphenanthrene has been amply confirmed by the production of tumours (seven epitheliomas and five papillomas in twenty mice) by 2-methyl-3:4-benzphenanthrene, which was synthesized by a slight modification of the new method.



2-Methyl-3:4-benzphenanthrene.

Fig. 8 shows that the activity of the new hydrocarbon is much higher than that of the parent 3:4-benzphenanthrene (compare fig. 1 of Part III), the appearance of tumours being more rapid than in the case of 1:2:5:6

dibenzanthracene (see figs. given in Part I). In fact, comparison with figs. 3 and 4 suggests an activity approaching that of methylcholanthrene. This high activity of a compound which, unlike the highly active compounds so far encountered, is not derived from 1:2-benzanthracene, is of considerable interest, and it is evident that derivatives of 3:4-benzphenanthrene merit further attention.

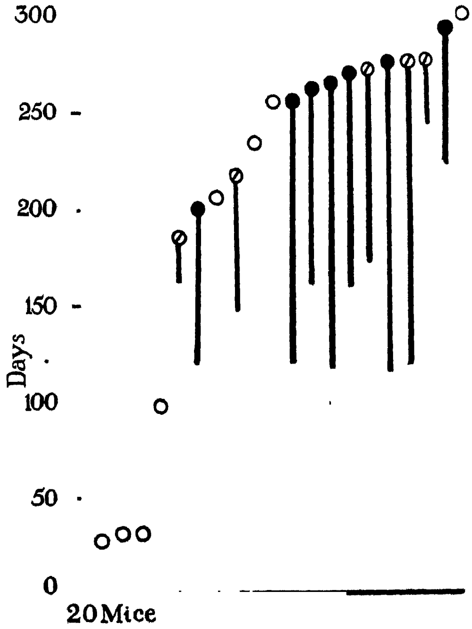
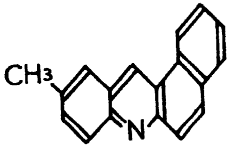
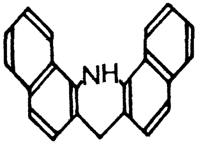
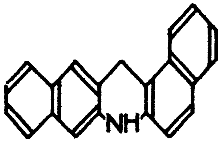


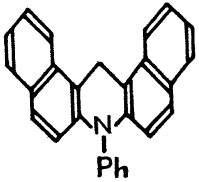
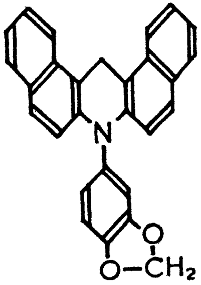
FIG. 8—2-Methyl-3 : 4-benzphenanthrene.

DIBENZACRIDINE DERIVATIVES

In view of the carcinogenic activity of two dibenzacridines structurally related to carcinogenic hydrocarbons (Part III) tests have been made with five dibenzacridine derivatives which were generously presented to us by Dr. W. O. Kermack. The results are summarized in Table V, from which it will be seen that no tumours have been obtained with any of these compounds; some of the mice are still living. It is perhaps unfortunate that Simpson mice only were used for testing two of these compounds, as it was subsequently observed (see p. 349) that in this strain of mice the skin shows delayed response to the highly carcinogenic hydrocarbon methylcholanthrene.

TABLE V—COMPOUNDS RELATED TO ACRIDINE

	Mice				Tumours	
		Alive after				
Compound	Initial number	6 months	1 year	Day of death of last mouse	Epithelioma	Papilloma
6-Methyl-3:4-benzacridine 	20 Simpson mice	18	8	373	0	0
9: 10-Dihydro-1:2:7:8-dibenzacridine 	20 ,,	9	7	2 alive, day 618*	—	—
9 : 10-Dihydro-3:4:6:7-dibenzacridine 	20 ,,	11	3	628	0	0
	10 Stock mice	10	7	5 alive, day 457*	—	—

10-Phenyl-9-10-dihydro-3:4:5:6-dibenzacridine 	10 Simpson mice 10 Stock mice 10 ,,	4 1 5	1 1 5	440 393 3 alive, day 520*	0 0 —	0 0 —
10-Piperonyl-9 : 10-dihydro-3:4:5:6-dibenzacridine 	20 Simpson mice 10 Stock mice	8 7	7 1	635 444	0 0	0 0

* 5 March 1937.

TUMOURS OF CONNECTIVE TISSUE PRODUCED BY 1:2:5:6-DIBENZACRIDINE

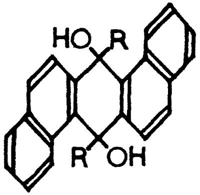
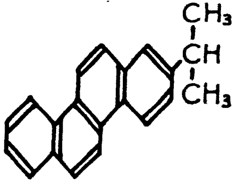
1:2:5:6-Dibenzacridine was dissolved in sesame oil (3 mg. in 1 c.c.; clear solution at room temperature) and the solution injected into the groin in nineteen mice (0·3 c.c.), usually at fortnightly intervals. Of thirteen of these mice which lived for more than 168 days eight developed, at the site of injection, spindle-celled tumours, of the type produced by other polycyclic compounds, between that date and the 240th day. These tumours did not grow well on transplantation into ordinary stock mice, and no one of them was carried beyond the sixth grafted generation.

3:4:5:6-Dibenzacridine was administered in the same way to two series of ten mice. In the first series, the last mouse died on the 310th day, and in the second series on the 266th day. No tumours have appeared in either series. Further experiments will be carried out to show whether there is actually a difference in sarcoma-producing power between these two compounds. When applied to the skin (Part III) the 3:4:5:6 compound was more effectual in producing epitheliomas than was the 1:2:5:6 compound.

SYNTHETIC OESTROGENIC COMPOUNDS

The possible influence of the oestrogenic hormones in promoting certain forms of cancer has been considered and investigated by numerous workers. In view of the emphasis which has sometimes been laid (although not by us) on the supposed close structural relationship between the oestrus-producing hormones and the carcinogenic hydrocarbons it seemed desirable to carry out tests for carcinogenic activity with highly active synthetic oestrogenic compounds which are much more closely allied in structure to the carcinogenic hydrocarbon, 1:2:5:6-dibenzanthracene. The most active synthetic oestrogenic compound so far known is 9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene, which has been applied to the skin of two series of twenty mice, of which all were males in one series. No tumours were obtained, although the duration of the experiment was such that very weak carcinogenic activity could not have been detected. Similar negative results were obtained with the corresponding di-*n*-butyl compound, which has an oestrogenic activity approaching that of the di-*n*-propyl compound (Table VI).

TABLE VI—OTHER POLYCYCLIC COMPOUNDS

Compound	Method of preparation	Mice: Initial no.	Mice: Alive after 6 months	Mice: Alive after 1 year	Mice: Day of death of last mouse	Tumours: Epi-thelioma	Tumours: Papil-loma
9:10-Dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene	Cook, Dodds, Hewett and Lawson (1934)	20♂	6	0	353	0	0
		20	16	2	458	0	0
9:10-Dihydroxy-9:10-di-*n*-butyl-9:10-dihydro-1:2:5:6-dibenzanthracene	Cook (1931)	10♂	2	1	622	0	0
		20	16	8	643	0	0
 [R=C_3H_7 *or* C_4H_9]							
Chrysene, synthetic	Cook and Hewett (1934)	20	11	6	853	0	1
4-*iso*Propylchrysene	Cook and Robinson (unpublished)	10	5	4	471	0	0
							
s-Triphenylbenzene	Reddelien (1913)	20	19	12	11 alive, day 450*	—	—
Tetraphenylmethane $C(C_6H_5)_4$		20	14	7	7 alive, day 457*	—	—
Pentaphenylethane, $Ph_3C.CHPh_2$		20	18	10	10 alive, day 443*	—	—
*iso*Naphthathioxin	Cohen and Smiles (1929)	10	9	3	438	0	0
Anthracene, synthetic		50	34	16	732	0	0
7:8-Dihydrophenalyl-7-*spirocyclo*-pentane	Cook and Hewett (1934)	30	25	14	567	0	0

* 5 March 1937.

OTHER COMPOUNDS

The question of the possible carcinogenic activity of chrysene was discussed previously (Part III). The test with the pure sample of synthetic chrysene has now been completed, and it is clear that this hydrocarbon has little or no carcinogenic activity towards the skin of mice. The synthetic compound was applied to the skin of twenty mice of which five lived for more than 440 days. The last mouse died on the 853rd day bearing a large epithelial tumour, which had appeared first on the 711th day; this showed some down-growth, but did not reach the superficial layer of voluntary muscle. No other mouse showed any tumour. No tumours were obtained in an experiment with 4-*iso*propylchrysene (Table VI).

Morton, Branch and Clapp (1936) have described the production of tumours with two hydrocarbons, tetraphenylmethane and *s*-triphenylbenzene, which differ widely in molecular structure from the condensed-ring compounds shown in this Institute to have cancer-producing properties. With tetraphenylmethane these authors obtained epitheliomas after 319 days in one strain of mice but not in another, and so far they had produced only sarcomas with triphenylbenzene. In attempting to secure confirmation of these results we have carried out experiments with the compounds in question and also with pentaphenylethane. The tetraphenylmethane and pentaphenylethane were prepared and highly purified by Professor M. Gomberg, to whom we are greatly indebted for the gift of these materials. Table VI shows that no tumours have been obtained in these experiments, which have now been in progress for about 14 months.

Tests with various compounds related to the sterols and the oestrogenic hormones were summarized in Table IX of Part III. Some of the mice in many of these experiments were still living, and the final results of these are now reproduced in Table VII, as although tumours have not been obtained with any of the compounds it seems desirable to indicate the duration of the experiments. In the case of the tests with the oestrogenic hormones and their derivatives these negative results merely record our failure to induce tumours of the skin by repeated application of the hormones. In this particular investigation we have not been concerned with the development of tumours at sites other than that of application of the compounds, and our results have no bearing on the question of the association of oestrogenic hormones with mammary cancer. We wish, however, to dissociate ourselves from any definition of carcinogenic action which limits the term to such agencies as can be shown to produce malignant changes in the skin. Obviously a compound which can produce

TABLE VII—STEROLS, OESTRINS AND RELATED COMPOUNDS

Compound	Method of preparation	Concen-tration % in benzene	Mice			
			Initial number	Alive after		Day of death of last mouse
				6 months	1 year	
Ergosterol		0·3	50	34	11	638
Ergosterol		0·3	20♀	18	18	784
Calciferol		0·3	20♀	15	11	703
Neoergosterol	Inhoffen (1932)	0·3	20	10	6	534
Oestrone: (1)		0·01	20♀	18	11	430
(2)		0·01	11♂C	10	6	705
Oestrone methyl ether: (1)		0·1	20♀	15	4	524
(2)		0·1	10♀C	5	2	507
(3)		0·1	20♂	12	0	332
(4)		0·1	6♂C	4	0	277
Oestriol		—	5	3	0	311
Pregnandiol (0·3 % in alcohol)		—	20	16	9	431
1:2-*cyclo*Pentenophenanthrene	Cook and Hewett (1933)	0·3	30	24	21	724
1:9-Dimethylphenanthrene	Haworth and Mavin (1932)	0·3	20	15	6	550
1-Keto-1:2:3:4-tetrahydrophenanthrene	Haworth (1932)	0·3	10♀	9	9	653

C = castrated.

cancer is entitled to be termed carcinogenic without any qualifying restriction as to the manner in which this is achieved.

[*Note added in proof* 19 *June* 1937.—It is now possible to give fuller reports of some experiments which were at an early stage when this paper was written.

(*a*) In the series of twenty mice treated with a solution of 3:4-benzpyrene in acetone (p. 353, and Table II) five mice are still living after 225 days. Of these, three have tumours which are probably epitheliomas, and one bears a papilloma. This establishes the point at issue, that the use of acetone as a solvent does not inhibit the carcinogenic action of benzpyrene.

(*b*) In a subsequent series of ten mice painted with 10-methyl-1:2-benzanthracene (p. 358) only three are still alive after 135 days. One of these bears a well-developed papilloma; the other two have no tumour.

(*c*) Out of three series of forty mice in all, which have been painted during 370 to 524 days with 3:4-benzphenanthrene (Table IV) six are now alive, of which three bear papillomas.

(*d*) The experiments with *s*-triphenylbenzene and tetraphenylmethane (Table VI) have now been in progress for 554 and 561 days respectively, without the production of tumours. Ten mice are still living in the former case, and five mice in the latter.

In addition, triphenylbenzene has been injected subcutaneously into a series of twenty mice, of which eight are living after 371 days, and tetraphenylmethane has been injected into twenty mice of which four are living after 363 days. No tumours have been obtained.]

We wish to record our indebtedness to the British Empire Cancer Campaign, and to the International Cancer Research Foundation, for generous grants which have supported this investigation. We are much indebted to Professor E. L. Kennaway, F.R.S., for his supervision of the animal experiments and for his histological diagnoses. We also wish to thank Miss M. Knox, B.A., for preparing the charts illustrating this paper, and for tabulating the records of experiments.

SUMMARY

The high carcinogenic potency of methylcholanthrene has not been surpassed with any other compound yet tested, but is almost equalled by that of cholanthrene.

New evidence is brought forward that the introduction of substituents

into the 5 position of the 1:2-benzanthracene molecule is a favourable condition for carcinogenic activity.

Three hexacyclic hydrocarbons derived from the pentacyclic carcinogenic hydrocarbon of coal tar, 3:4-benzpyrene, are very potent in producing epitheliomas in mice.

2-Methyl-3:4-benzphenanthrene has cancer-producing activity of a high order. This is one of the few known carcinogenic compounds not related to 1:2-benzanthracene.

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Description of Plates

Epithelial tumours produced in rats by application of methylcholanthrene to the skin.

Plate 14

Fig. 9—Rat *A*. Day 461. Downgrowth from epidermis resembling rodent ulcer. × 62·5. (R. 2075).

Fig. 10—Rat *A*. Portion of skin adjacent to fig. 9. Downgrowth from epidermis having alveolar arrangement. × 100.

Plate 15

Fig. 11—Rat *B*. Day 657. Downgrowth from epidermis showing squamous (right) and non-squamous (left) portions. × 35. (126/35).

Fig. 12—Rat *B*. Columnar arrangement of cells just below epidermis. × 141.

547·963·4 : 546·173

The Action of Nitrite on Haemoglobin in the Absence of Oxygen

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(*Communicated by D. Keilin, F.R.S.—Received* 23 *April* 1937)

Much work has been done on the action of nitrite on haemoglobin,* but there is little agreement on the course of the reaction. In the presence of a reducing agent Haldane (1901; Haldane and others 1897) detected only the formation of NO-haemoglobin (the compound formed by the action of nitric oxide on reduced haemoglobin); in the presence of oxygen a mixture of methaemoglobin and NO-haemoglobin was apparently obtained (cf. Haurowitz 1924; Meier 1925). On the other hand, Hartridge (1920–1) believed that in the presence of oxygen one compound, nitrite-methaemoglobin, was formed. The constitution of NO-haemoglobin, usually assumed to be a ferro-compound of the same type as oxyhaemoglobin or CO-haemoglobin, is also doubtful. Anson and Mirsky (1925) state that NO-haemoglobin is a ferri-compound, the first product of the reaction between NO and haemoglobin being methaemoglobin which then combines with NO.

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* A review of earlier work is given by Meier (1925).

The presence of oxygen probably complicates the reaction between nitrite and haemoglobin. If the mechanism involves HNO_2 the presence of oxygen may result in an ill-defined concentration of NO. In addition, although the oxidation of haemoglobin to methaemoglobin by oxygen is relatively slow (Brooks 1931, 1935) nitrite may act as catalyst. Finally, in the presence of oxygen and excess nitrite compounds other than methaemoglobin and NO-haemoglobin are certainly formed (Meier). For these reasons the reaction was studied quantitatively in the absence of oxygen.

MATERIAL AND METHODS

Sodium nitrite was added to haemoglobin in a phosphate buffer (*a*) in the absence of oxygen, (*b*) in the presence of a reducing agent. The Hartridge reversion spectroscope was used to identify and estimate the products; later a gasometric method was found which confirmed the spectroscopic results.

Sodium Nitrite. A.R. $NaNO_2$ was twice recrystallized and solutions were freshly prepared before use.

Buffer Mixtures. The reaction mixtures were buffered with K_2HPO_4 and KH_2PO_4; the total concentration of phosphate was 0·1 M. The *p*H was obtained from the data of Cohn (1927) and Green (1933) and by the glass electrode.

Reducing Agents. Sodium hydrosulphite and titanous tartrate solutions were prepared immediately before use. The 4 % $Na_2S_2O_4$ solution contained enough NaOH or NH_4OH to make the *p*H roughly 7. The titanous tartrate solution (Conant, Scott and Douglass 1928) was made by adding 0·4 c.c. 15 % titanous chloride solution (B.D.H.) to 1·6 c.c. of a solution saturated with sodium potassium tartrate and 0·2 M with respect to H_3BO_3 and 1·8 M with respect to KOH.

Haemoglobin. Laked blood and solutions of pure haemoglobin were used. Defibrinated pig's blood was laked by several freezings and thawings. Pure haemoglobin was prepared from pig's blood by the method of Adair and Adair (1934). Both solutions were stored at −20° C. and portions thawed when required. The manometric apparatus of van Slyke and Neill (1924) was used to measure the following concentrations in the thawed solutions before use:

(1) Haemoglobin—from the CO capacity determined by the method of van Slyke and Hiller (1928).

(2) Total pigment—from the CO capacity after reduction with $Na_2S_2O_4$ determined by the method of van Slyke and Hiller (1929).

(3) Undenatured pigment—from the O_2 capacity after reduction with titanous tartrate determined by a slight modification of the method of Conant, Scott and Douglass (1928).

The concentration of haemoglobin is given by (1), methaemoglobin by (3) minus (1), and denatured haemoglobin by (2) minus (3). There was never any denaturation during storage; there was, however, a very slow oxidation of haemoglobin to methaemoglobin at − 20° C. Analyses of a solution of pure haemoglobin after preparation are given below. The results are expressed in vols. %, i.e. c.c. CO or O_2 (0° C., 760 mm. Hg) combined with or liberated from the pigment contained in 100 c.c. The concentration in equivalents per litre is given by (vols. %) × 10/22,400 (a gram-molecule of haemoglobin containing four equivalents):

(1) Haemoglobin	28·93, 28·99 vols. %
(2) Total pigment	30·12, 30·12 vols. %
(3) Undenatured pigment	30·03, 30·25 vols. %.

It will be seen that during preparation about 4 % methaemoglobin was formed but there was no denaturation.

Nitric oxide. NO was prepared in an all-glass apparatus by the method of Noyes (1925).

Oxygen-free nitrogen. Cylinder N_2 was freed from O_2 by the method of Kautsky and Thiele (1926).

The experiments were usually carried out at roughly 15° C.; the same final values were obtained at 0° C.

SPECTROSCOPIC RESULTS

Comparison of NO-*haemoglobin and nitrite-haemoglobin*

Haldane's result was confirmed by comparing (1) the positions of the mid-points of the two absorption bands of NO-haemoglobin with (2) those observed when excess $NaNO_2$ was added to a haemoglobin solution containing $Na_2S_2O_4$.

(1) Excess NO was admitted into the evacuated vessel.* *A* (fig. 1) containing (*a*) laked blood† diluted in a phosphate buffer, (*b*) pure haemo-

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* With the Hartridge spectroscope available it was difficult to obtain accurate readings unless a flat-sided vessel was used.

† The blood was used immediately after slaughter to avoid methaemoglobin formation during storage.

globin in a phosphate buffer containing 0·1 % $Na_2S_2O_4$ (any methaemoglobin present or formed was therefore changed to reduced haemoglobin). The vessel was evacuated (with a Cenco pump) and filled with O_2-free N_2 three times, followed in the case of (*a*) by shaking the solution *in vacuo* for 30 min. Oxygen was previously removed from the NO-generating vessel *B* and its reagents by the passage of N_2. The long thin-walled capillary *C* allowed *A* to be shaken. The position of the bands was the same in (*a*) and (*b*). No prior formation of methaemoglobin was observed in (*a*), none could occur in (*b*) (cf. Anson and Mirsky 1925). Similar results have recently been obtained by Keilin and Hartree (1937).

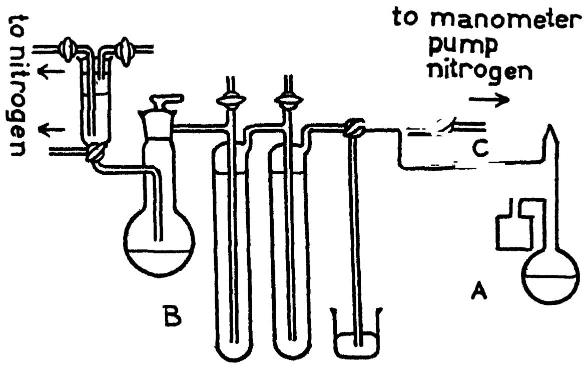

FIG. 1—Action of NO on a haemoglobin solution.

(2) Excess $NaNO_2$ was added to laked blood or pure haemoglobin in a phosphate buffer containing 0·1 % $Na_2S_2O_4$. Mixing was first carried out in a sealed vessel filled with N_2 but this was found to be unnecessary; it was sufficient to cover the mixture with a thick layer of liquid paraffin. The positions of the only visible bands are given in Table I.

TABLE I

Band	NO-haemoglobin A	Excess $NaNO_2$ added to haemoglobin in the presence of $Na_2S_2O_4$ A
α-band	5753	5750
β-band	5371	5369

The values are the mean of several experiments; different values varied by less than 3 A. The position of the bands was independent of the *p*H (6·22–7·75). The only detectable product of the action of nitrite on haemoglobin in the presence of $Na_2S_2O_4$ is apparently NO-haemoglobin.

The action of nitrite on haemoglobin in the presence of $Na_2S_2O_4$

In these and other experiments laked blood and pure haemoglobin gave the same results and no further distinction is made between them.

A calibration curve for mixtures of reduced haemoglobin and NO-haemoglobin was obtained with the usual double-wedge trough technique. The mixtures showed one broad, rather ill-defined band;* the mid-point of this merged band for different proportions of the two pigments is given in fig. 2. The same curve was given at different *p*H values. It was difficult to measure the position of the band accurately and each value is the mean of twelve readings.

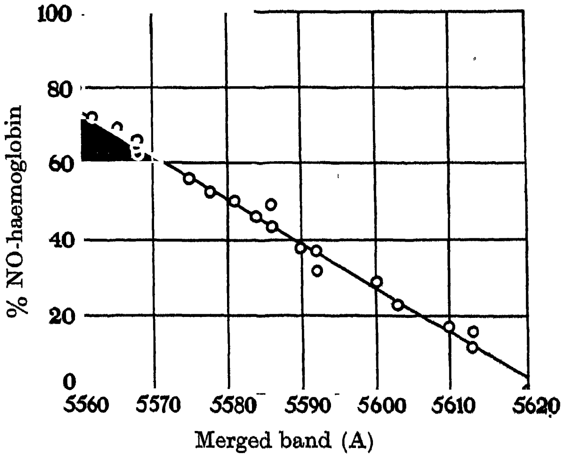

Fig. 2—Position of merged band of reduced haemoglobin and NO-haemoglobin.

A solution of $NaNO_2$ was added to haemoglobin in a phosphate buffer containing 0·1 %† (or more) $Na_2S_2O_4$. After covering the mixture with paraffin the position of the merged band was measured at intervals until a final value was obtained. Some results at *p*H 6·22 are given in Table II; each value is the mean of at least six experiments.

Within the limits of the rather large experimental error 1 mol. $NaNO_2$ changed completely one equivalent reduced haemoglobin to NO-haemoglobin in the presence of $Na_2S_2O_4$. The same result was obtained over the *p*H range 6·22–6·63, and at *p*H 6·22 when the concentration of haemoglobin was increased twentyfold (thinner flat-sided cells were used for the higher concentrations).

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* At points corresponding to very high proportions of NO-haemoglobin the α- and β-bands could be distinguished on the background of the single broad reduced haemoglobin band.

† Increasing the concentration of $Na_2S_2O_4$ above this value had no effect on the results obtained.

TABLE II—2×10^{-6} EQUIVALENTS REDUCED HAEMOGLOBIN IN 10 C.C. BUFFER; *p*H 6·22; 15° C.

Mol. $NaNO_2$ added		Position of band A	NO-haemoglobin %	Equivalents NO-haemoglobin formed
0		5620	0	0
$0 \cdot 5 \times 10^{-6}$		5603	25	$0 \cdot 5 \times 10^{-6}$
1·0		5581	50	1·0
1·5		5560	75	1·5
2·0	α-band	5750	100	2·0
	β-band	5370		

The rate of reaction decreased with increasing *p*H and was relatively very slow at *p*H 7·16–7·75. Over this range final values were not obtained in the presence of an equivalent amount of $NaNO_2$* in 6 hr. at 15° C.

For other reasons similar measurements were made using a phosphate buffer containing NaCl. The total concentration of phosphate was 0·1 M and the ratio $[H_2PO_4^-]/[HPO_4^=]$ was 4. A decrease in *p*H with increasing ionic strength would be expected. Values of the *p*H, determined by the glass electrode, are given in Table III; they can be represented approximately by the following equation (cf. Cohn 1927; Green 1933):

$$pH = 7 \cdot 20 - \frac{1 \cdot 5\sqrt{\mu}}{1 + 1 \cdot 5\sqrt{\mu}} + K_s\mu - \log\frac{[H_2PO_4^-]}{[HPO_4^=]},$$

where μ is the ionic strength and K_s is given the value $-0 \cdot 27$.

TABLE III

Concentration of NaCl %	Ionic strength	*p*H observed	*p*H calculated
0	0·14	6·22	6·20
5	1·00	5·66	5·73
10	1·85	5·38	5·43
15	2·71	5·15	5·15

The same result was obtained in each buffer mixture—complete change of one equivalent reduced haemoglobin to NO-haemoglobin by 1 mol. $NaNO_2$ in the presence of $Na_2S_2O_4$. The rate of reaction increased with decreasing *p*H (or increasing salt concentration). The times for 50 % change of reduced haemoglobin to NO-haemoglobin with the experimental conditions used were very roughly 10, 4 and 1 min. at *p*H 5·66, 5·38 and 5·15 respectively and 15° C.

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* At *p*H 7·75 reduced haemoglobin is changed to NO-haemoglobin completely in the presence of excess $NaNO_2$.

The action of nitrite on haemoglobin in the absence of a reducing agent

In these experiments no reducing agent was used; oxygen was removed by evacuation. The haemoglobin solution contained therefore reduced haemoglobin and a small amount of methaemoglobin (present initially in the stock solution).

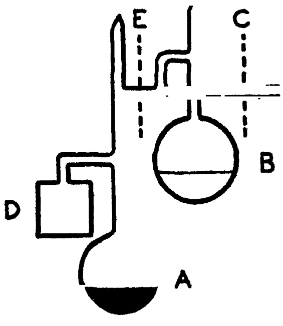

Fig. 3—Vessel used for mixing solutions of nitrite and haemoglobin.

Bulb *A* (fig. 3) contained haemoglobin in a phosphate buffer, bulb *B* an aqueous solution of $NaNO_2$. After evacuating and filling with N_2 three times the solutions were shaken *in vacuo* for 30 min. and the vessel sealed off at *C*. After mixing the solution was transferred to the cell *D*.

The following bands were finally observed at *p*H 6·22 on adding $2 \cdot 18 \times 10^{-6}$ mol. $NaNO_2$ to $4 \cdot 36 \times 10^{-6}$ equivalents reduced haemoglobin ($0 \cdot 10 \times 10^{-6}$ equivalents methaemoglobin were initially present but not detectable spectroscopically*):

Band A	Remarks
6327	Rather faint band
5752	—
5366	—
ca. 4910	Ill-defined band

The two outer bands have the same position as the α- and δ-bands of methaemoglobin (pig's methaemoglobin, *p*H 6·22) and the two inner bands as the α- and β-bands of NO-haemoglobin.† Methaemoglobin and NO-haemoglobin were therefore the only detectable products (the α-band of methaemoglobin was also observed by Meier when nitrite acted on haemoglobin in an atmosphere of hydrogen).

The presence of methaemoglobin was confirmed by the addition of $Na_2S_2O_4$. The two outer bands disappeared and a broad merged band

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* Before mixing, the solution in *A* was transferred to *D* and no methaemoglobin bands were visible.

† Using the double-wedge trough and solutions of NO-haemoglobin and methaemoglobin it was found that the position of these bands was the same at different points along the trough. The β- and γ-bands of methaemoglobin were not visible with the thickness of cell and the concentrations used.

(reduced haemoglobin plus NO-haemoglobin) appeared at 5580 A, corresponding to 50 % of each compound (fig. 2). Roughly therefore $2\cdot18 \times 10^{-6}$ mol. $NaNO_2$ formed $2\cdot23 \times 10^{-6}$ equivalents NO-haemoglobin and $2\cdot13 \times 10^{-6}$ equivalents methaemoglobin (since NO-haemoglobin is stable in the presence of $Na_2S_2O_4$).

With smaller relative amounts of $NaNO_2$ two methaemoglobin bands and a merged band of NO-haemoglobin and uncombined reduced haemoglobin were seen. Let the position of the merged band correspond to a fraction x of NO-haemoglobin in the mixture of NO-haemoglobin and reduced haemoglobin. Methaemoglobin was then changed to reduced haemoglobin by the addition of $Na_2S_2O_4$; let the position of the new merged band correspond to a fraction y of NO-haemoglobin. If the initial amount of reduced haemoglobin plus methaemoglobin was a, and the initial amount of methaemoglobin was b equivalents, then:

$$\text{Equivalents NO-haemoglobin formed} = ay,$$
$$\text{Equivalents methaemoglobin formed} = a - b - ay/x.$$

The results of several experiments analysed in this way are given in Table IV, the pH was 6·22.

TABLE IV

Initial amount total pigment $= 4\cdot46 \times 10^{-6}$ equivalents
Initial amount methaemoglobin $= 0\cdot10 - 0\cdot25 \times 10^{-6}$ equivalents

Mols. $NaNO_2$ added	Equivalents NO-haemoglobin formed	Equivalents methaemoglobin formed
$2\cdot18 \times 10^{-6}$	$2\cdot23 \times 10^{-6}$	$2\cdot13 \times 10^{-6}$
1·36	1·26	1·50
1·20	1·21	1·30
1·09	1·09	0·90

Roughly therefore 1 mol. $NaNO_2$ gives one equivalent NO-haemoglobin and one equivalent methaemoglobin. It would be expected that the values in the last column would be less consistent than those in the second.

Gasometric Results

As spectroscopic analysis was rather unsatisfactory owing to the lack of definition of the bands another method, using van Slyke's manometric technique, was found.

With a mixture of NO-haemoglobin, reduced haemoglobin and methaemoglobin, the CO or O_2 capacity gives the concentration of reduced haemoglobin and the CO capacity after reduction with $Na_2S_2O_4$ (or the O_2 capacity after reduction with titanous tartrate) gives the sum of the concentrations of reduced haemoglobin and methaemoglobin if it can be assumed that NO-haemoglobin or a derivative is inert (as regards gas evolution or uptake) during both determinations. The determinations include an equilibration with CO or O_2 and the action of an acid $K_3Fe(CN)_6$ reagent.

The inertness of NO-haemoglobin to acid $K_3Fe(CN)_6$* was first established. To $3{\cdot}67\times10^{-5}$ equivalents reduced haemoglobin in a phosphate buffer containing $Na_2S_2O_4$ was added $3{\cdot}67\times10^{-5}$ mol. $NaNO_2$. When only NO-haemoglobin was detectable spectroscopically, 2 c.c. of the mixture (total volume 14·5 c.c.) were analysed by the usual method for total pigment, except that shaking *in vacuo* for 5 min. was substituted for shaking with 150 mm. CO. A value of 0·18 vol. % was obtained; the control (from an analysis of a solution of the same concentration of phosphate and $Na_2S_2O_4$ but containing no haemoglobin or nitrite†) was 0·16 vol. %. The initial concentration of reduced haemoglobin in the mixture was 5·67 vol. %. Although NO-haemoglobin was precipitated in some unknown form there was no gas evolution or uptake. A similar result was obtained using titanous tartrate in place of $Na_2S_2O_4$.

It was found that shaking for 5 min. with 150 mm. CO apparently changed about 23 % of the NO-haemoglobin present to CO-haemoglobin, this combined CO being liberated on adding acid $K_3Fe(CN)_6$. As haemoglobin has a much smaller affinity for O_2 than for CO or NO, air was then used for equilibration; with 5 min. shaking there was no detectable decomposition of NO-haemoglobin. A mixture of NO-haemoglobin, reduced haemoglobin and methaemoglobin can therefore be analysed (if the total concentration of the three pigments is known) by determining:

(i) the O_2 capacity—reduced haemoglobin,

(ii) the O_2 capacity after reduction with titanous tartrate‡—reduced haemoglobin plus methaemoglobin.

The action of nitrite on haemoglobin in the presence of titanous tartrate

In these experiments titanous tartrate was used in place of $Na_2S_2O_4$ in the reaction mixture. When no further change was observed spectroscopically 2 c.c. of the mixture, 3 drops caprylic alcohol and 4·7 c.c. H_2O were shaken with air for 5 min. in the chamber of the van Slyke apparatus with the mercury meniscus at the 50 c.c. mark and the tap open. The air was expelled and 0·3 c.c. acid $K_3Fe(CN)_6$ reagent added. After shaking *in vacuo* for 5 min. any CO_2 present was absorbed by the admission of 1 c.c. air-free NaOH and the residual gas measured in the usual way.* The details of one experiment are given below:

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* The more acid reagent of van Slyke and Hiller (1929) was used: 92 c.c. 32 % $K_3Fe(CN)_6$ solution, 20 c.c. lactic acid (sp. gr. 1·2).

† Free $NaNO_2$ gives a gas evolution; $3{\cdot}67\times10^{-5}$ mol. $NaNO_2$ in 14·47 c.c. phosphate buffer (containing $Na_2S_2O_4$) analysed as for total pigment gave a value of 2·06 vol. %, corresponding to 54 % of the total NO which would be liberated if the reaction $3HNO_2 \rightarrow H^+ + NO_3^- + 2NO + H_2O$ went to completion.

‡ $Na_2S_2O_4$ cannot be used when the O_2 capacity after reduction is to be determined since the oxidation products of $Na_2S_2O_4$ markedly accelerate the oxidation of haemoglobin to methaemoglobin by oxygen. Titanous tartrate is free from this objection (Conant, Scott and Douglass 1928).

Total phosphate 0·1 M; *p*H 6·41; 15° C.; 7·74 c.c. reaction mixture contained initially:

1 c.c. titanous tartrate solution,
$3{\cdot}36 \times 10^{-5}$ equivalents reduced haemoglobin,
$1{\cdot}74 \times 10^{-5}$ mol. $NaNO_2$.

Analyses (O_2 capacity) after 2½ hr. standing under paraffin:

(*a*) 4.63 vol. %.
(*b*) 4·66 vol. %.

Equivalents uncombined reduced haemoglobin

$$= \frac{4{\cdot}65 \times 7{\cdot}74}{100 \times 22400} = 1{\cdot}61 \times 10^{-5}.$$

$$\text{Equivalents NO-haemoglobin formed} = (3{\cdot}36 - 1{\cdot}61) \times 10^{-5}$$
$$= 1{\cdot}75 \times 10^{-5},$$
$$\text{Mol. NaNO}_2 \text{ added} = 1{\cdot}74 \times 10^{-5}.$$

Results are summarized in Table V; each value is the mean of duplicate determinations.

TABLE V—*p*H 6·18–6·58; 15° C.

Mol. $NaNO_2$ added	Equivalents reduced haemoglobin initially present	Equivalents NO-haemoglobin formed
$3{\cdot}91 \times 10^{-5}$	$3{\cdot}91 \times 10^{-5}$	$3{\cdot}88 \times 10^{-5}$
1·96	3·91	2·03
1·00	3·91	0·98
1·74	3·36	1·75
1·00	3·52	1·01

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* Equilibrating with 760 mm. air in the chamber of the van Slyke apparatus gives a large value for the control (about 6 vol. %) but this can be determined very accurately by repeating the whole of the procedure (omitting haemoglobin and nitrite from the reaction mixture but not the titanous tartrate) and standardizing rates and times of shaking etc. A correction factor for the effect of temperature on the value of the control was also determined.

The results agree with those described previously; in the presence of a reducing agent 1 mol. $NaNO_2$ gives one equivalent NO-haemoglobin.

The action of nitrite on haemoglobin in the absence of a reducing agent

In these experiments no reducing agent was used. A similar vessel to that shown in fig. 3 was used (the flat-sided cell was absent) and oxygen was removed by evacuation etc. After sealing off at *C* and mixing (*in vacuo*) the solution was distributed between bulbs *A* and *B* and the vessel was divided in half by sealing off at *E*. One-half was used for duplicate determinations of the O_2 capacity as described in the previous section, the other for the O_2 capacity after reduction with titanous tartrate. In the second case 2 c.c. of the mixture, 3 drops caprylic alcohol, 4·3 c.c. H_2O and 0·4 c.c. titanous tartrate solution were shaken with air for 5 min. in the chamber of the van Slyke apparatus; the rest of the analysis has been described.*

The details of one experiment are given below. Two corrections were necessary: (*a*) separate experiment showed that about 2 % of the haemoglobin was oxidized to methaemoglobin† during evacuation; (*b*) there was a decrease in volume caused by evaporation during evacuation, this was measured by weighings.

Total phosphate 0·1 M; *p*H 6·22; 15° C.

Amounts of pigment present before adding $NaNO_2$:

Total pigment = $6{\cdot}72 \times 10^{-5}$ equivalents.

Reduced haemoglobin = $5{\cdot}86 \times 10^{-5}$ equivalents.

Methaemoglobin = $0{\cdot}86 \times 10^{-5}$ equivalents ($0{\cdot}12 \times 10^{-5}$ formed during evacuation).

Total volume of solution after mixing = 17·22 c.c.

Analyses:

(1) O_2 capacity 0·22, 0·18 vol. %.

(2) O_2 capacity after reduction with titanous tartrate 4·95, 5·01 vol. %.

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* This is essentially a slight modification of the method of Conant, Scott and Douglass (1928) for undenatured pigment. The preparation of the titanous tartrate solution in an atmosphere of N_2 was found to be unnecessary. It was also more convenient to equilibrate the reduced solution in the chamber of the van Slyke apparatus than in a tonometer.

† The rate of oxidation of haemoglobin to methaemoglobin by oxygen increases with decreasing oxygen pressure (Brooks 1935).

After adding $NaNO_2$:

$$\text{Equivalents reduced haemoglobin} = \frac{0{\cdot}20}{100 \times 22400} \times 17{\cdot}22$$

$$= 0{\cdot}15 \times 10^{-5}.$$

Equivalents reduced haemoglobin plus methaemoglobin

$$= \frac{4{\cdot}98}{100 \times 22400} \times 17{\cdot}22$$

$$= 3{\cdot}83 \times 10^{-5}.$$

$$\text{NO-haemoglobin formed} = (6{\cdot}72 - 3{\cdot}83) \times 10^{-5}$$

$$= 2{\cdot}89 \times 10^{-5} \text{ equivalents.}$$

$$\text{Methaemoglobin formed} = (3{\cdot}83 - 0{\cdot}15 - 0{\cdot}86) \times 10^{-5}$$

$$= 2{\cdot}82 \times 10^{-5} \text{ equivalents.}$$

$$NaNO_2 \text{ added} = 2{\cdot}90 \text{ mol.}$$

Results are summarized in Table VI; each value is the mean of duplicate determinations.

TABLE VI—*p*H 6·22–6·63; 15° C.

Mol. $NaNO_2$ added	Equivalents NO-haemoglobin formed	Equivalents methaemoglobin formed
$1{\cdot}44 \times 10^{-5}$	$1{\cdot}46 \times 10^{-5}$	$1{\cdot}43 \times 10^{-5}$
1·63	1·60	1·64
2·00	2·04	1·99
2·90	2·89	2·82

It will be seen that 1 mol. $NaNO_2$ gives one equivalent NO-haemoglobin and one of methaemoglobin.

DISCUSSION

Conant and his co-workers (1923, 1924–5, 1926, 1928, 1932) have shown that reduced haemoglobin and methaemoglobin form a reversible oxidation-reduction system of similar type to the ferrocyanide-ferricyanide system. The change of haemoglobin to methaemoglobin requires one hydrogen-equivalent of oxidizing agent per gram-atom of iron in the haemoglobin molecule (i.e. per one equivalent haemoglobin). If reduced haemoglobin is represented by $(H_3X)_4$, methaemoglobin is then $(H_2X)_4$.* With the conditions of pH, etc., used 1 mol. $NaNO_2$ in the absence of oxygen or a reducing agent gives one equivalent NO-haemoglobin and one of methaemoglobin, i.e. the reaction goes to completion and no nitrate is formed. It follows that the stoichiometric equation for the reaction can be written:

$$2\,(H_3X)_4+4HNO_2\rightarrow(H_3X.NO)_4+(H_2X)_4+4H_2O, \tag{1}$$

where $(H_3X.NO)_4$ represents NO-haemoglobin. The results show clearly that NO-haemoglobin is a ferro-compound of the same type as oxyhaemoglobin or CO-haemoglobin since the one hydrogen equivalent of oxidizing agent available per mol. of HNO_2 is fully accounted for by the production of one equivalent methaemoglobin.

In the presence of a reducing agent methaemoglobin is changed to reduced haemoglobin and the net reaction can be written:

$$(H_3X)_4+4HNO_2+2R\rightarrow(H_3X.NO)_4+2H_2O+2RO, \tag{2}$$

where R represents the reducing agent and RO its oxidation product.

The increase in the rate of reaction with decreasing pH observed qualitatively suggests that the mechanism involves HNO_2. Abel and his co-workers (1928, 1930) have shown that the decomposition of HNO_2 is given by the net equation:

$$3HNO_2\rightarrow H^++NO_3^-+2NO+H_2O \tag{3}$$

and consists of two steps:

$$4HNO_2\rightleftharpoons N_2O_4+2NO+2H_2O,\quad \text{rapidly established equilibrium,} \tag{4}$$

$$N_2O_4+H_2O\rightarrow HNO_2+H^++NO_3^-,\quad \text{rate-determining step.} \tag{5}$$

Since no nitrate is formed in the reaction between nitrite and haemoglobin in the absence of oxygen it appears that the net reaction (3) can be neglected. This is consistent with the range of pH and temperature used. Kinetic measurements would be necessary to determine the reaction mechanism; a possible one is as follows:

$$\left.\begin{aligned}8HNO_2&\rightleftharpoons 2N_2O_4+4NO+4H_2O,\\4NO+(H_3X)_4&\rightarrow(H_3X.NO)_4,\\2N_2O_4+3(H_3X)_4&\rightarrow(H_3X.NO)_4+2(H_2X)_4+4H_2O.\end{aligned}\right\} \tag{4}$$

* The representation of reduced haemoglobin and methaemoglobin by $(H_3X)_4$ and $(H_2X)_4$ respectively is similar to the convention used by Conant and Fieser (1924–5). It shows formally the difference in valence of the iron-containing radicals in the 2 mol.

A study of the action of N_2O_4 on reduced haemoglobin would be interesting in this connexion.

The results throw no light on the reaction in the presence of oxygen. It is clear that NO-haemoglobin would not be the sole product, but the question whether derivatives other than NO-haemoglobin and methaemoglobin are formed is still unanswered.

SUMMARY

The reaction between $NaNO_2$ and haemoglobin in the absence of oxygen was studied spectroscopically and gasometrically. Both methods gave the same results.

In the presence of a reducing agent a compound is formed with the same absorption bands as NO-haemoglobin prepared by the action of NO on reduced haemoglobin.

There was no prior formation of methaemoglobin in the reaction between NO and reduced haemoglobin.

In the presence of a reducing agent 1 mol. $NaNO_2$ changed completely one equivalent reduced haemoglobin to NO-haemoglobin at *p*H 5·15–6·63. The rate of reaction decreased with increasing *p*H and was relatively very slow at *p*H 7·16–7·75.

In the absence of both oxygen and a reducing agent 1 mol. $NaNO_2$ combined with two equivalents reduced haemoglobin to give one equivalent NO-haemoglobin and one equivalent methaemoglobin.

The results show that NO-haemoglobin is a ferro-compound of the same type as oxyhaemoglobin and CO-haemoglobin.

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A Graphical Solution of a Differential Equation with application to Hill's Treatment of Nerve Excitation

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I—Graphical Analysis

Value of the Method

Linear differential equations with constant coefficients are very common in physical and chemical science, and of these, the simplest and most frequently met is the first-order equation

$$a\frac{dy}{dt}+y=f(t), \tag{1}$$

where a is a constant, and $f(t)$ a single-valued function of t. The equation signifies that the quantity y is removed at a rate proportional to the amount present at each instant, and is simultaneously restored at a rate dependent only upon the instant in question. Familiar examples of this equation are the charging of a condenser, the course of a monomolecular reaction, the movement of a light body in a viscous medium, etc.

The solution of this equation is easily shown to be

$$y = e^{-t/a}\left\{y_0 + \frac{1}{a}\int_0^t e^{t/a} f(t)\, dt\right\}, \tag{2}$$

where y_0 is the initial value of y. In the case where $f(t) = 0$, this reduces to the well-known exponential decay of y.

The expression (2) is usually not hard to evaluate when $f(t)$ is given in the form of a simple algebraic function, but in cases where this does present difficulties, and in the large class of cases where $f(t)$ is supplied in the form of an experimental curve, a graphical method of solution has advantages.

The ordinary method of evaluating

$$\int_0^t e^{t/a} f(t)\, dt$$

by plotting the integrand against t and finding the area between the curve, the axis of t, and the ordinate at various values of t, has two objections. In the first place it is very tedious to find the area for a large number of values of t. But more serious, if we are concerned with values of t/a which are at all large, the value of $e^{t/a}$ increases so enormously that the curve will not lie on the paper.

The method to be described derives the results immediately in the form of the curve y as function of t without any computation, and thus the solution may readily be obtained by those unfamiliar with integration and the analytical treatment of these equations.

The method may be extended to equations of second or higher orders in those cases where the solution is non-oscillatory (or more precisely when the auxiliary equation has real roots), since such equations can always be reduced to a succession of first-order equations. In the present paper, however, we shall consider only certain cases of the first and second orders.

Nature of the Analysis

In order to perform this analysis we need a simple appliance which may be constructed from a piece of card in the course of a few minutes. A more accurate and durable model will readily suggest itself to those interested.

Cut from a piece of firm card the slot AB (fig. 1*a*) 10–15 cm. long and of width just so much more than 5 mm., that a pencil will draw a line of exactly 5 mm. in passing perpendicularly across the slot. Make a mark O at about the middle of the left edge, and obtain the point P on the line from

O perpendicular to AB such that $OP = 25$ mm. exactly. Cut out a piece of card CD which has a straight edge in such a position that the pencil line drawn against it will, if produced, pass through a point Q. Pass a drawing pin through P from below, so that the point sticks up; pass the point through Q; finally place a small piece of cork over the pin and press well down to shield the point and to make a firm pivot, which will allow CD to rotate about P.

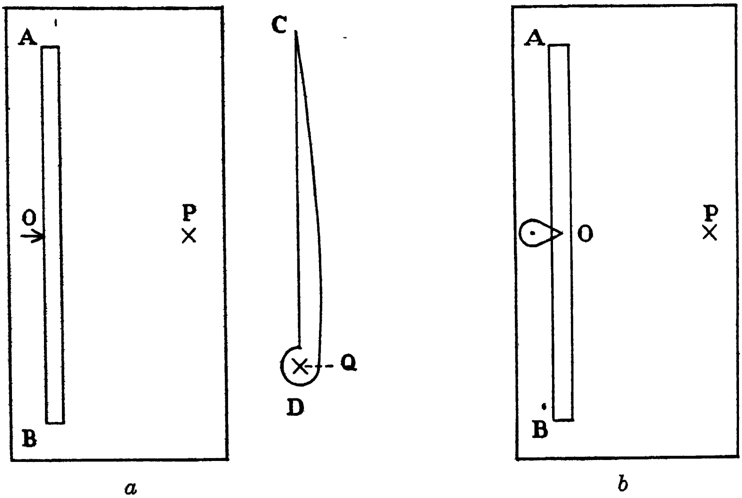

FIG. 1—*a*, diagram of analyser, see text; *b*, more accurate model.

The analyser is now ready for use.

We are given the curve $f(t)$ (fig. 2) and also y_0, the initial value of y. The ordinate scale is arbitrary, being the same both for $f(t)$ and for y, but the time scale is very important.

In equation (1) a is seen to have the dimension of time, and the curve of $f(t)$ must be plotted upon such a scale that a corresponds to the length OP of the analyser which we have made 25 mm.

The curve is analysed as follows:

Place the analyser over the curve with AB parallel to the axis of ordinates, and with P to the right of O (i.e. not upside down). Place a pencil point vertically on y_0 in the left edge of the slot AB of the analyser, and move the latter so that O coincides with the first point of the curve $f(t)$, i.e. $f(O)$. Swing the straight edge CD round to meet the pencil, and hold CD firm while the pencil moves along it across to the right edge of the slot drawing an oblique line. Now, without moving the pencil, shift the analyser 5 mm. to the right, i.e. until the left edge of the slot engages the pencil; see that

AB is still vertical, and slide vertically up or down so that *O* still lies over the curve $f(t)$. The curve which the pencil traces out step by step is the required curve of y.

It is convenient to have a name for the foregoing analysis, and since this is based upon the property that the subtangent of an exponential curve is constant, the name "Subtangent Analysis" will be used.

This account must appear tedious and difficult, but manipulations are invariably far easier to perform than minutely to describe, and if the reader cares to carry out the above descriptions, he will find that a suitably scaled curve can be analysed in about 2 min.

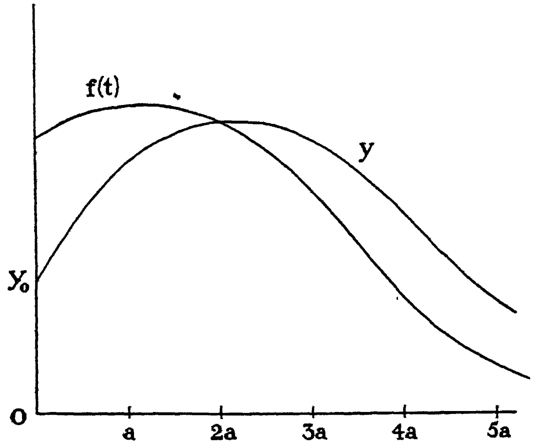

FIG. 2—Abscissae: time in units of a, the constant of the analyser. Given $f(t)$ and y_0, required to obtain the curve of y, from equation (1).

When greater accuracy is required a slight modification may be introduced in the analyser. Instead of *O* being as shown in fig. 1*a*, it is made the tip of a pointer jutting into the middle of the slot *AB* as shown in fig. 1 *b*. *P* is also moved to the right so that *OP* is the same length as before.

In this way the short straight lines of which the derived curve is composed are theoretically exact near their mid-points instead of at their left ends. This centring eliminates certain cumulative errors.

Method Employed when a *is Large*

In the foregoing method of analysis it has been assumed that it is convenient so to scale the axis of time that the constant a may be represented by a length of 25 mm. This is not always the case. For instance, we shall treat later in this paper cases where two analyses have to be performed in succession with very different constants. In these cases it is more convenient to use two analysers, with the same curve, than to replot the curve on a different scale, especially if many curves have to be so treated. But if we have to use two analysers with the distance OP differing in the ratio (say) 20 : 1, it is clear that the smaller will be too minute, or the larger too clumsy for accuracy. In such a case the best method is to use the smaller analyser as already described, and for the larger, proceed as follows.

Two sheets of paper are taken similarly ruled in squares. Upon the first is drawn the given curve $f(t)$ with large ordinate scale, upon the second is drawn a pair of axes to receive the curve traced out by the analysis. The two sheets are pinned on to a drawing board so that the first lies to the right of the second, the two horizontal axes are collinear, and the two origins separated by the distance a exactly.

Now take a straight edge somewhat longer than a, having a mark, O, near the right-hand end. Place O on the first point of the curve $f(t)$ and swing the edge round so that it passes through the point y_0 on the left-hand sheet. From y_0 draw a line along the edge for one unit to the right (say 1 cm.). Now move O one unit to the right, keeping it on the curve $f(t)$, and, adjusting the edge to meet the last point of the line just drawn, draw from here another unit. In this way the curve may be analysed with no apparatus but a straight edge.

The point O on the straight edge merely serves the purpose of marking the place on the curve $f(t)$ where the contact with the edge is being made. Without this indicator, one is constantly losing the place, which must be found by counting or measurement before the next step in the analysis can be performed.

Mathematical Justification

Fig. 3 represents the operation of the analyser, and O, P and C correspond to the same letters in fig. 1a. As O follows the given curve $f(t)$, the line PC is a tangent to the derived curve y.

So that

$$\frac{dy}{dt}=\frac{f(t)-y}{a},$$

or

$$a\frac{dy}{dt}+y=f(t),$$

and y is the solution of equation (1).

This assumes that the analysis is performed in infinitely small steps. In practice, steps should be small enough for there to be no significant change in the curve obtained when the size of each step is halved. It is legitimate to vary the size of the step during the course of an analysis in order to treat critical regions more minutely.

It is obvious that this analysis may also be applied conversely in order to obtain $f(t)$ when the curve of y is given.

In this case the edge CD, fig. 1, is set tangential to the given curve of y at the point where the vertical AB cuts it, and a mark is made where the point O lies. The curve joining successive marks made in this way is the required curve $f(t)$ as follows from the geometry of fig. 3.

Used in this way the analyser is a crude replica of the accurate instrument designed by Keith Lucas (1912) for the analysis of capillary electrometer records.

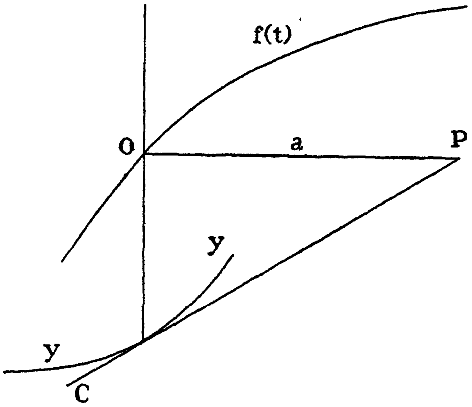

FIG. 3—Proof of the validity of the subtangent analysis. Abscissae: time (see text).

II—APPLICATION TO HILL'S TREATMENT OF NERVE EXCITATION

In a series of recent papers Hill and his co-workers have published the results of stimulating nerves in many different ways. The experiments have been conducted with great precision and the results have been found to fit accurately a formula derived from some rather general physical considerations which are set forth in a theoretical exposition (Hill 1936).

According to this analysis, the stimulating current, I, of any form produces in the nerve a change in the "local potential" V, which is defined by Hill's equation (4).

Differentiating with respect to t, and putting V_1 for $(V-V_0)$, we obtain

$$k\frac{dV_1}{dt}+V_1=kbI. \tag{3}$$

For very brief currents it is necessary and sufficient that V attain some value U_0 for excitation to occur, but with currents of longer duration the process of "accommodation" enters, and V must attain a value U which is greater than U_0 and defined by Hill's equation (7) which, upon differentiating and putting U_1 for $(U-U_0)$, becomes

$$\lambda\frac{dU_1}{dt}+U_1=V_1. \tag{4}$$

Thus if we are given I it is mathematically possible to obtain first V_1 from (3), and then U_1 from (4), and hence to find the conditions for excitation to occur, namely that V_1-U_1 should attain some fixed value which may be taken as unity without loss of generality.

The practical difficulty arises in the evaluation of V_1 and U_1 from equations (3) and (4), and we proceed to a critical comparison of three methods, and to a more detailed account of one method in particular, which has the advantage that solutions may rapidly be obtained from any given curve of I by a graphical method which may be carried out as a routine without any mathematical knowledge.

Evaluation of V_1 and U_1

Three methods are available, computation, models, and graphical analysis. Computation is on the whole the most satisfactory when the worker is familiar with integrals, and when I may be written down as an analytical expression which can be integrated; this is the method adopted by Hill.

Models have the advantage that no mathematical knowledge is required and they give the results ready plotted as a curve, but they may be inaccurate and troublesome to construct and employ.

Monnier (1934) constructed the circuit shown in fig. 4, where the stimulus was applied across the low resistance AB, and V_1-U_1 is represented by the potential difference DE, recorded by an oscillograph. Another model giving the same result, but representing a little more closely what may be taking place in the tissue, is shown in fig. 5, where C_1 is a condenser which has to attain a critical charge for excitation to occur, and C_2 is a series condenser representing some interposed polarisable structure. For those who have an oscillograph these models are simple and accurate, and have only the disadvantage that a circuit must actually be constructed to give the required stimulus before its effect can be recorded, and sometimes this may be difficult as in the case of an exponentially increasing current of the form

$$I=I_0e^{\beta t},$$

which is not easy to produce experimentally, but which plays an important part in the theory of nerve conduction.

Graphical Analysis

The graphical analysis described above is easy to perform, and will solve equations such as (3) and (4). This "subtangent analysis" will now be applied to one or two cases of excitation to illustrate the method by which Hill's curves may be readily obtained without any mathematical working.

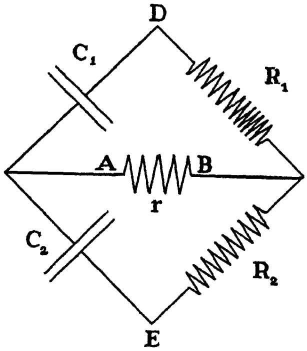

FIG. 4—Monnier's electrical model.

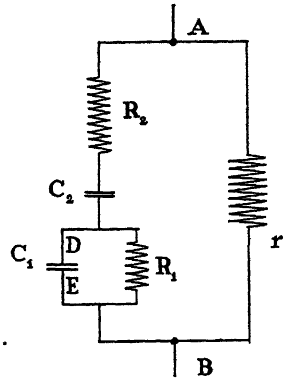

FIG. 5—An alternative circuit.

We have seen that the equation

$$a\frac{dy}{dt}+y=f(t)$$

may be solved by performing a certain graphical operation called "subtangent analysis" upon the curve $f(t)$ using a subtangent of a. Hence the solution of V_1 is obtained at once from (3) by performing the analysis upon the given curve kbI using a subtangent of k. Again, the value of U_1 is immediately found by analysing this derived curve with subtangent λ. This procedure is applicable to any and every case, and may be employed in a routine fashion.

Practical details are as follows:

Details of the analysis. kbI is plotted on paper ruled in squares. The vertical scale is made rather large, and the horizontal time scale such that k corresponds to a distance of from 1 to 2·5 cm. A piece of tracing paper is pinned over the graph, and upon this the first analysis is carried out with subtangent k and with the first point of the derived curve zero. A mark is made on the curve at each cm. distance from the vertical axis. Now the tracing paper is unpinned and carried to the right a distance equal to λ exactly, and fixed there with the time axis collinear with that of the given curve. Now the curve on the tracing paper (which is V_1) is analysed with subtangent λ by the method applicable to large subtangents, and this will result in a second curve being drawn on the same sheet as I with the first point at the origin. This is the curve of U_1. It merely remains to unpin the tracing paper, and superpose it again upon the first sheet. If the axes are made to coincide, the ordinate difference between the two curves corresponding to any instant is easily shown to be inversely proportional to the current strength required for excitation to occur at that instant. For if I, the current strength, is increased n times, the vertical scale of the whole analysis is increased n times, and hence the ordinate difference $(V_1 - U_1)$ is also thus increased. And since $(V_1 - U_1)$ must attain unity to excite, this condition will now be obtained at the instant where formerly $(U_1 - V_1) = 1/n$.

Condenser Discharge

As example take a condenser discharge, fig. 6, where S is the curve representing the stimulus. When this is analysed with subtangent k we obtain V_1, and this again analysed with λ gives U_1. In the example quoted the ratio λ/k is 8, which is a case of very fast accommodation, and therefore one that shows up conspicuously in the figure. If we are concerned with values of λ slower than this, there will be little error in reducing all the ordinates of U_1 in the same proportion, which should be inversely as λ.

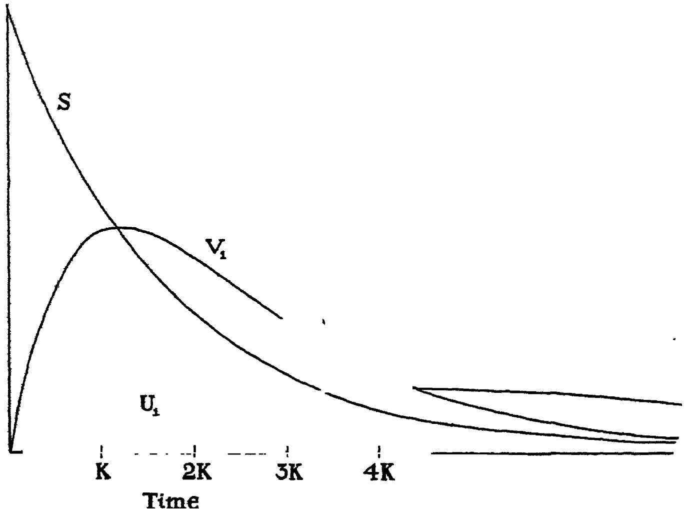

FIG. 6—Analysis of condenser discharge of time constant 1·8k. $\lambda/k = 8$.

This is admissible only if we are concerned with the interval during which V_1 is increasing, at longer times the error is much greater.

Threshold excitation will occur (if it does) at the instant when the ordinate difference $(V_1 - U_1)$ is maximal. This is easiest found by sliding the tracing paper containing V_1 vertically downward (so that the vertical axes still coincide) until the curve V_1 no longer cuts but merely touches U_1. Then the point of contact gives the moment of excitation, and the distance between the two origins is inversely proportional to the required stimulus.

If the origins are farther apart, the current is subthreshold and the two curves never meet. If the origins are closer, the current is above threshold, and excitation occurs earlier, namely, at the first point of intersection of the two curves.

It is seen that this analysis gives curves from which the threshold may at once be related to duration of flow, hence it is particularly useful in those cases where the current is cut off before it has produced its maximum effect. In condenser discharges, where the current is usually allowed to flow to completion, it is necessary to make a separate analysis for each discharge rate, though obviously it is unnecessary to analyse further than the point where V_1 is maximal.

It is not difficult to calculate the voltage-capacity relation for stimulation with condenser discharges of various rates, on the assumption that accommodation is absent (Rushton 1931, p. 272; Blair 1932; Hill 1936), but when accommodation is taken into account the computations become very heavy. The problem may, however, be solved relatively easily by the application of subtangent analysis in the foregoing manner to a representative selection of exponential curves. The threshold voltage obtained in each case is then plotted against the capacity corresponding to the particular exponential analysed.

Linearly Increasing Currents

The case where $$I = \alpha t$$ lends itself more readily, perhaps, to graphical than to analytical treatment, especially if only qualitative results are required, for these may be obtained at a glance.

In fig. 7 are shown the three curves as usual, namely the given kbI which in this instance is $kb\alpha t$, and the curves of V_1 and U_1. V_1 will obviously start horizontal, since the pivot of the analyser is initially on the axis, and clearly U_1 will run nearly horizontal for a distance still greater. It is also apparent that the two derived curves will ultimately coincide with the dotted lines which run parallel to *kbαt*, and which cut the time axis at k and $(k+\lambda)$ respectively.

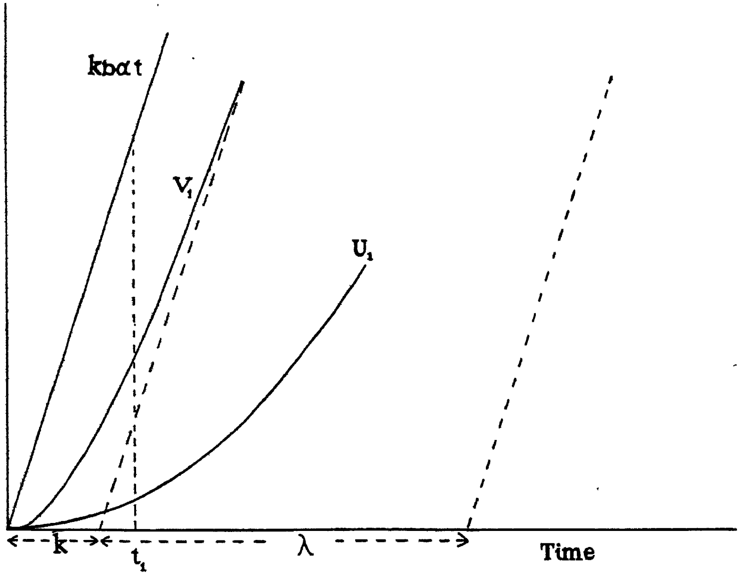

FIG. 7—Analysis of linearly increasing current *kbαt*. The curves V_1 and U_1 become asymptotic to the dotted lines parallel to *kbαt*.

Now the maximum *ordinate* difference between V_1 and U_1 will be the ordinate difference between the two dotted lines, which is the gradient $kb\alpha$ multiplied by the horizontal separation λ, and since for excitation to occur $V_1 - U_1$ must be unity we have the condition for the minimum effective gradient

$$kb\alpha\lambda = 1.$$

Now if I_0 is Hill's "true rheobase", i.e. the rheobase when $\lambda = \infty$, it is easy to see that

$$kbI_0 = 1.$$

Hence the minimum gradient α has the value

$$I_0/\lambda.$$

Now, if the rate of increase of the stimulus is greater than the minimum effective one, excitation will occur earlier, and at any instant t_1 (fig. 7), the intensity of the current at the moment of stimulation will be given by the ordinate drawn at t_1 to the line *kαbt* compared with the ordinate difference

$(V_1 - U_1)$ at t_1 which must be taken as unity if excitation is to occur at that instant.

This ratio
$$\frac{kb\alpha t}{V_1 - U_1}$$

is seen to be infinity when $t_1 = 0$. As t_1 increases the ratio sinks to a value which, when k/λ is small (instead of 1/4 in the present instance), becomes very nearly unity. For in that case the ordinate V_1 will become large compared to the ordinate difference $(kb\alpha t - V_1)$ at a value of t where U_1 has not yet become appreciable. For large values of t the ratio

$$\frac{kb\alpha t}{V_1 - U_1}$$

rises again indefinitely as $kb\alpha t$ continues to increase while $(V_1 - U_1)$ remains constant. The actual relation is plotted in fig. 9 of Hill's (1936) paper (ordinates multiplied by 1·8).

DISCUSSION

The graphical analysis which has been described is an easy and reliable method of predicting the excitatory value of any given type of stimulus, and when any known form of stimulus is given, such as the condenser discharge in the first example, the threshold strength can readily be found in terms of, say, the "true rheobase" I_0 and the two constants k and λ. When we have a form of stimulus which is cut off before being allowed to flow to completion, as in the second example, the method allows us to determine after a single construction the relation between the duration of current flow and the intensity of the particular form of stimulus.

There is no object in pursuing further examples since the same method can be applied to each case, and all the principal results have already been given by Hill (1936).

One point, however, which has already been mentioned by Hill, needs emphasis. The two quantities V_1 and U_1 of this analysis are merely convenient constructions in the solution of our problem, and may well have no independent physical significance.

All that we are concerned with in excitability measurements is the difference $(V_1 - U_1)$ which we may call θ, and the fundamental equation of excitation is

$$\frac{d^2\theta}{dt^2} + \left(\frac{1}{k} + \frac{1}{\lambda}\right)\frac{d\theta}{dt} + \frac{\theta}{k\lambda} = b\frac{dI}{dt}, \tag{5}$$

which involves a single variable, but of course the two time constants k and λ.

In the solution of this second-order equation it is convenient (but by no means necessary) to resolve it into the difference of two first-order equations, and these two equations are (1) and (2) at the beginning of this paper and their solutions V_1 and U_1.

But the separate existences of V_1 and of U_1 cannot be justified by any conceivable excitability measurements so long as they obey the equations under consideration, for in that case all measurements enter only as the single variable

$$V_1 - U_1 = \theta.$$

The matter is well illustrated in the electrical models shown in figs. 4 and 5.

If θ is the potential difference across DE, I is the potential difference across AB, and r is the resistance of AB, then from the arrangement in fig. 4 we obtain

$$\frac{d^2\theta}{dt^2} + \left(\frac{1}{C_1R_1} + \frac{1}{C_2R_2}\right)\frac{d\theta}{dt} + \frac{\theta}{C_1C_2R_1R_2} = r\left(\frac{1}{C_1R_1} + \frac{1}{C_2R_2}\right)\frac{dI}{dt},$$

which shows that the model satisfies equation (3) with C_1R_1 and C_2R_2 equal to k and λ.

The arrangement in fig. 5 on the other hand gives

$$\frac{d^2\theta}{dt^2} + \left(\frac{1}{C_1R_1} + \frac{1}{C_2R_2} + \frac{1}{C_1R_2}\right)\frac{d\theta}{dt} + \frac{\theta}{C_1C_2R_1R_2} = \frac{r}{R_2}\cdot\frac{dI}{dt},$$

which also satisfies equation (3), but now the values of k and λ each involve all the constants $C_1C_2R_1R_2$ in a more complicated way, so that the quantities V and U in this case have no simple physical significance.

Since both the foregoing models satisfy the same equation (3) they equally represent all the phenomena of excitation which are governed by it, and if we are to decide between them (and between many other models which may be made to give the same result) we must use criteria other than excitability measurements.

For the investigation of excitability, however, any analysis of (3) will serve equally well, and the method which is most convenient is that here described, which, though involving V_1 and U_1, implies nothing concerning the physical existence of either of these quantities.

SUMMARY

A method is described of solving graphically the equation

$$a\frac{dy}{dt}+y=f(t).$$

It is quick, simple, and may easily be performed by those unfamiliar with integration.

The method is particularly suitable when $f(t)$ is given in the form of an experimental curve rather than an algebraic expression.

By performing this "subtangent" analysis n times in succession, linear differential equations of the nth order may be solved, provided that all the roots of the auxiliary equation are real.

This analysis is applied to the solution of Hill's treatment of nerve excitation (1936), and a simple routine operation is described which will give the curve relating current strength with duration of flow for any form of stimulating current.

The application is illustrated by the case of a condenser discharge and of linearly increasing currents.

The physical significance of V and U is discussed.

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Discussion Meeting
on
The Transmission of Excitation in Living Material

3 *June* 1937

Opening Address

By C. F. A. Pantin, F.R.S.

on

JUNCTIONAL TRANSMISSION OF STIMULI IN THE LOWER ANIMALS

In the vertebrates, graded response of skeletal muscle depends on the multiplicity of motor units. As Gaskell (1920) pointed out, in other animals each muscle is often supplied by the repeated division of only a very few axons, or even by a single motor axon. There is evidence here that gradation of response is effected chiefly through the properties of the neuromuscular junction, and the problem has been studied extensively in Crustacea and coelenterates. A single stimulus is usually ineffective or only causes a trivial response, but it leaves an effect which facilitates the transmission of a second impulse to the muscle. Each impulse in a series facilitates the transmission of excitation to more and more muscle fibres (Pantin 1935 and 1936; Katz 1936*a*).

The facilitating effect of a stimulus is maximal during its relative refractory period. It gradually dies away. Thus for stimuli repeated at short intervals the number of muscle fibres brought into action rapidly increases, giving a large quick contraction, while at long intervals the facilitating effect of each stimulus decays so much before the next arrives that the contraction is small and develops slowly.

It seems likely that neuromuscular facilitation is common. There is evidence for it in the Mollusca and the echinoderms as well as arthropods and coelenterates. There is an evident analogy with the condition of vertebrate skeletal muscle during partial curarization (Adrian and Lucas 1912).

A widespread phenomenon of this kind must be taken into account in any theory of transmission. There are two views concerning transmission at neuromuscular and other junctions. According to one, excitation is directly transmitted by the action current; according to the other, the impulse liberates some transmitter substance which in turn excites the muscle fibre. The facilitation phenomena at first suggest a chemical transmitter, but Bremer (1930) and Monnier (1936) have shown how electrical phenomena accompanying the nervous impulse may account for many similar facts with equal suitability. Certain evidence suggests that the decay at least of the facilitation is due to the removal of some chemical substance, rather than to the decay of an electrical condition set up by an action current. The time taken for the facilitation process to decay is very long compared with the refractory period. It persists after one would have expected the electrical state to return to normal. Again, experiments on Actinozoa show that while facilitation and excitability are profoundly influenced by the ionic composition of the medium, yet the rate of decay of facilitation is remarkably unaffected, though the ionic changes might reasonably be supposed to affect the electric state of the tissues. On the other hand, there is every reason to suppose that if a chemical transmitter is involved, it is not acetylcholine (Bacq 1935, 1937). Both Crustacea and sea anemones are very sensitive to changes of potassium concentration, and it is possible that potassium plays some part in transmission.

At present there is not enough evidence to prove either the electric or the chemical hypothesis for facilitating systems. But there are facts which help to define the problem. In summarizing their conclusions on transmission in vertebrate systems, Dale, Feldberg and Vogt (1936) suggest alternative hypotheses:

(1) That the impulse directly excites the muscle cell but that it cannot reach it unless the latter is sensitized by the chemical substance produced at the nerve ending.

(2) That a transmitter substance released at a nerve-ending directly excites the muscle cell.

These authors favour the latter hypothesis. Monnier has brought together evidence in favour of a double mechanism for transmission which might include Dale's first hypothesis. In Crustacea and sea anemones, the evidence favours the first hypothesis.

It is characteristic of sea anemones that a single stimulus normally causes no response. A contraction begins only with the second stimulus following the facilitating effect of the first. A simple chemical hypothesis would suggest that a single stimulus produces insufficient transmitter to reach the threshold. The second stimulus would add sufficient to cause a contraction. Now facilitation can be greatly modified in several ways. Mg can depress it to vanishing point, K and Ca can greatly exaggerate it.

Despite this, the first stimulus remains ineffective and the response begins at the second. If the effect were due to a chemical transmitter normally produced below threshold quantity, one would expect that enhancing facilitation must make the first stimulus effective, while depressing it with Mg should delay the appearance of contraction beyond the second stimulus. This does not happen. On the other hand, the peculiar relation found is to be expected from Dale's first hypothesis. The first stimulus fails in this case not because the transmitter is below threshold, but because the first impulse does not find the neuromuscular junction prepared for transmission.

Finally, in both anemones and Crustacea the contraction is built up by responses of the muscle directly corresponding to each stimulus received. As Katz points out, if the facilitation process is due to progressive accumulation of a chemical excitor substance, one would expect that after many stimuli the amount of transmitter would become so great that numerous after-discharges would appear between the stimuli, and this is not the case. The phenomena of facilitation all favour the view that some cumulative process at the nerve endings facilitates the transmission of impulses, but does not excite the muscle itself, and the wide distribution of facilitation suggests that other kinds of transmission are related to it.

A. v. Muralt (*Bern*)—*Observations on Chemical Wave Transmission in Excited Nerve*

In the attempt to isolate any possible "action substance" (corresponding to action current) occurring in the propagated chemical wave in an excited nerve, special attention has to be paid to the time relations. It is to be expected that "action substances" will appear and disappear in the nerve structure within the same time as the electrical phenomena, i.e. in milliseconds. If their liberation is closely connected with the action current there will also be no possibility of accumulating them by repetitive stimulation.

In our experiments liquid air was used for rapid and efficient fixation of the excited state in a peripheral nerve. Considerable shortening of the freezing process and control of the velocity was effected by an apparatus, with which nerves can be shot into liquid air at a constant and well-known velocity (fig. 1).

Method—Two symmetrical nerves (sciatics of *Rana esculenta* or other nerves) are prepared and mounted in turn in the apparatus. One nerve is stimulated by just maximal condenser discharges (method of Hill 1932) in a well-shielded electrode, care being taken that no fluid can run down the nerve from the stimulated spot. The part of nerve on and close to the electrode is always discarded, excluding therefore the objections which Gaddum, Khayyal and Rydin (1937) have brought forward against the

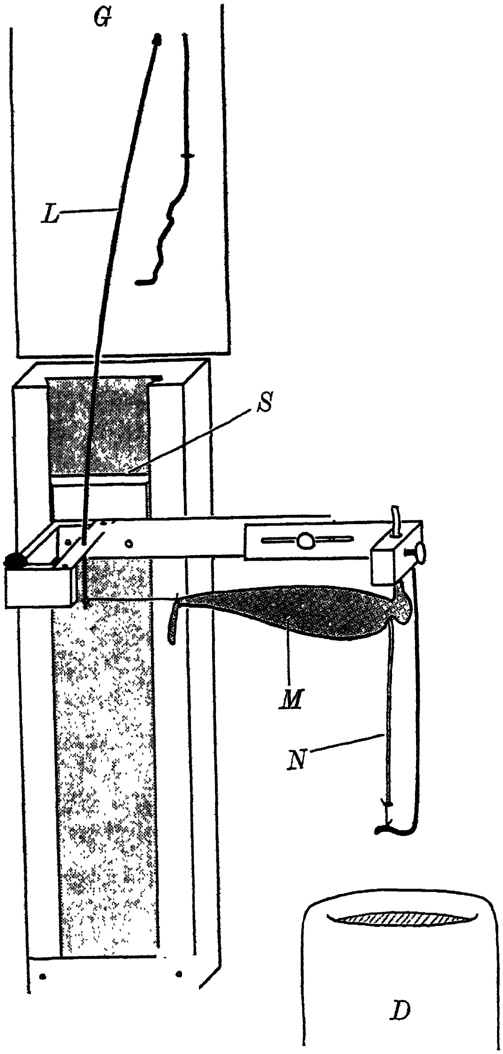

FIG. 1—Nerve-muscle preparation mounted on isometric lever in a sliding apparatus, set into motion by a falling weight dropping down at constant velocity. (The weight and the arrangement which stops the motion are not drawn.) *N*, nerve; *M*, muscle; *L*, lever writing the response on smoked glass surface *G*; *D*, Dewar flask with liquid air; *S*, sliding arrangement.

experiments of Binet and Minz (1936), Calabro (1933), and Bergami (1936). While stimulation goes on, the nerve is shot into the liquid air and kept there. The symmetrical nerve is placed into the electrode and treated in the same way, except for stimulation. Both nerves are then minced separately in solid CO_2 until a fine grain is obtained. For the study of pharmacologically active substances 10 c.c. of eserine Ringer (1 : 200,000) is added to each nerve; for the study of changes in the phospholipids 10 c.c. of CCl_4.

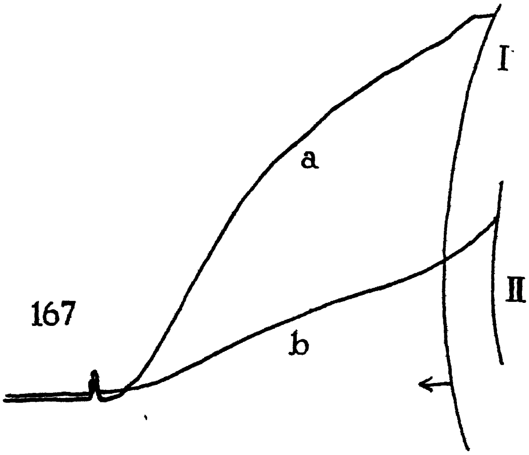

FIG. 2—Response of eserinized leech dorsal muscle to 10 c.c. eserine Ringer (1 : 200,000) extract from frozen nerve. *a*, right nerve stimulated; *b*, left nerve unstimulated. 17 May 1937.

Filtered extracts of nerves, tested 3 or 4 min. after addition of eserine Ringer, show a difference between "stimulated" and "unstimulated" side in their action on the eserinized leech muscle. The pharmacologically active substance is unstable in alkali (10 min.), has no action on normal leech muscle, and disappears in extracts on boiling and also on standing at room temperature (in about 2–3 hr.). A typical record of the active substance is given in fig. 2. Calculated as "A.C. equivalent" (Chang and Gaddum 1933), the amount obtained from "stimulated" nerve corresponds to 0·2 μg./g. of substance and from "unstimulated" nerve 0·12 μg./g. There is thus a noticeable increase due to stimulation with condenser discharges.

"Shooting" a nerve into liquid air without electrical stimulation does not exclude the possibility of excitation of the nerve at the spot of contact with the liquid air. The "unstimulated" control nerve might therefore have been excited as well. The response of a nerve-muscle preparation was therefore studied with the aid of the apparatus shown in fig. 1, when the nerve was shot into the liquid air at various speeds. If the velocity of shooting in is 130 cm./sec. no response is obtained. Apparently at this speed the conduction velocity in the cooled area above the liquid air is so slow that the excitation wave arising from the contact with liquid air is not able to escape. At 65 cm./sec. in 20 % of the experiments conduction took place and a response was obtained, at 5 cm./sec. all nerves gave conducted responses.

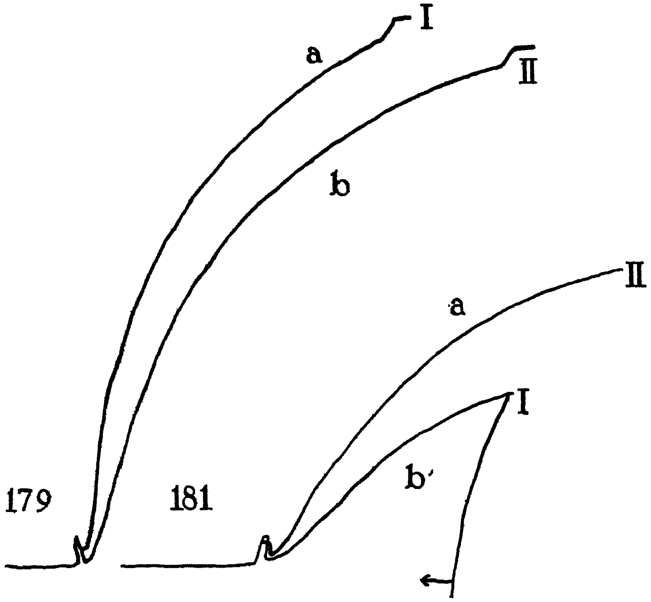

Fig. 3—Response of eserinized leech dorsal muscle to 10 c.c. eserine Ringer (1 : 200,000) extract from nerves "shot" into liquid air on unpolarizable silver-silver chloride electrode. *a*, catelectrotonus; *b*, anelectrotonus.

With a velocity of 130 cm./sec. no conduction takes place, which does not exclude that excitation still occurs while the nerve is frozen. In order to see whether the pharmacologically active substance appearing also in "unstimulated" nerve was due to local excitation from the liquid air the excitability of the nerves was altered by electrotonus. One nerve was shot into the air in "catelectrotonus", the other nerve in "anelectrotonus". Two typical records are shown in fig. 3. (Exp. 181 was performed on the two halves of one nerve, Exp. 179 on two symmetrical nerves.) The experiment shows that apparently the contact with the liquid acts in the same way as an electric stimulus (although there is no conducted response) producing a greater amount of "A.C. substance" when the nerve is more excitable. It shows also that there is a certain amount of "A.C. substance" present that has nothing to do with excitation.

A. L. HODGKIN—*Evidence for Electrical Transmission of the Nervous Impulse*

The experiments to be described were designed to test the theory that nervous impulses are transmitted by electric currents. The method employed consisted in observing the effect of a nervous impulse upon the region immediately beyond a localized block. It was found that an impulse which arrived at a cold or compression block caused a transient lowering of the electrical threshold in the nerve beyond the block. In freshly blocked nerve the decrease in threshold amounted to between 50 and 90%. According to an electrical theory the explanation of this effect would be that the eddy currents set up by the action potential can spread through the block and increase excitability in the nerve beyond. Fig. 4 shows how this could occur. If the flow of current beyond the block was just below threshold, an impulse would not traverse the block, but it would produce a large increase in excitability.

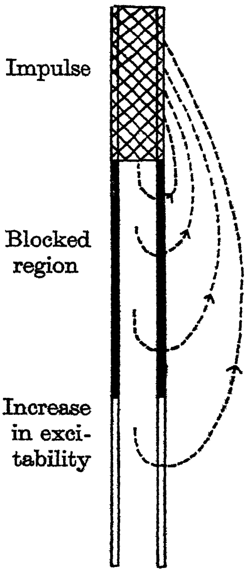

FIG. 4

The existence of these eddy currents can be proved experimentally. When the electrical changes are examined, it is found that the increase in excitability is invariably accompanied by a characteristic potential change. This has the same form as an action potential, but it declines exponentially along the nerve beyond the block. Measurements of the distribution of the potential show that it is of an electrotonic nature. This is to be expected, since the eddy currents postulated in the conventional membrane theory are essentially the same as those which are assumed to account for electrotonic spread.

The occurrence of the electrotonic potential suggests that it is the cause of the increase in excitability. This conclusion is borne out by a number of observations. Thus the time relations of the potential and excitability are very similar. This is strikingly illustrated by experiments with pressure blocks. Here the main electrotonic wave is usually followed by a large negative after-potential and in these cases there is always a corresponding "tail" in the excitability curve.

Further evidence for the electrotonic origin of the increase in excitability comes from a study of its spatial distribution. If the excitability and potential are measured at various points beyond the block, it is found that the change in threshold falls off with distance at about the same rate as the electrotonic potential.

The agreement in spatial and temporal properties shows that the spread of electrotonic current must be the cause of the change in threshold. This result strongly supports an electrical theory of transmission. For we should expect that the electrotonic currents immediately in front of an active region would be much more effective than those which spread through a block. Therefore, since a blocked action potential can produce a 90% decrease in threshold, it is extremely probable that the electrotonic currents associated with a normal impulse would be easily sufficient to excite. This conclusion is strengthened by the fact that the threshold electrotonic potential seems to be small compared to the action potential. In freshly blocked nerve a potential spread of 1 mV causes a 30–40% decrease in threshold, which suggests that 2–3 mV would be sufficient to excite. Now in these nerves the amplitude of the action potential is about 20 mV, so that the spike is nearly ten times greater than the threshold potential. Since the action potential is such an effective stimulus, it seems reasonable to conclude that the electrical theory is correct, and that the transmission of impulses depends upon the eddy currents which spread in front of the active region.

FRITZ BUCHTHAL (*Copenhagen*)—*Potential Differences in the Single End-plate-Muscle Fibre System, their relation to Irritability and their Changes under Excitation*

Measurements of potential differences (p.d.) in the single muscle fibre and in the transmitting system, end-plate-muscle fibre, were made in order to have conditions more exactly defined than is possible on the whole muscle and in order to study the relationship between structural and functional properties, these potentials being used as indicators of the state of the system. In examining the single fibre-end-plate system conditions become less complicated, and it is possible not only to trace the changes of p.d. under excitation and contraction, but also to make reproducible observations on the p.d. in the *uninjured* resting system (Buchthal 1937*a*). The numerous alternative electrical paths, the insulator and capacity properties of fascies and sarcolemma, conceal these potentials and their structural relations when measurements are made on the whole muscle.

If two non-polarizable micro-electrodes are placed about 100–200μ apart on the same muscle fibre, a p.d. of 2–4 mV is obtained, its magnitude being proportional to the distance between the electrodes (Buchthal and Péterfi 1934). Thereafter, keeping the same distance, the electrodes are moved in such a way that the grid electrode is put on a motor end-plate (lizard muscle fibre), and the earthed electrode on the corresponding fibre. A potential difference can now be measured which is about ten times as high as that measured with both electrodes on the fibre (electrostatic valve voltmeter). These potentials on the surface of the uninjured living system remain constant for several hours. The p.d. of injured fibres is inconstant and decreases considerably in a few minutes, while structure and birefringence undergo distinct changes and both indirect and direct irritability disappear. The temperature coefficients of injury potentials and of the p.d. measured on the uninjured surface of the fibre are distinctly different (Buchthal and Lindhard 1936).

The motor end-plate is a morphologically and functionally specialized region of the fibre (Lindhard 1931). Since the potential differences might be a suitable indicator for irritability and excitation of the transmitting system, an analysis of the relation between p.d. and reactivity was attempted, in collaboration with Professor Lindhard (Buchthal and Lindhard 1934 and 1935).

Poisoning with *curare* causes the end-plate-fibre potential to decrease. In about 10–30 min. it has disappeared so that only the resting potential of the muscle fibre is measured, the end-plate acting merely as a continuation of the one electrode to the fibre. In the same way *indirect irritability* of the fibre disappears, and only by using a direct stimulus can a contraction be brought about. In some cases the curare effect upon p.d. and irritability was *reversible*, both being restored to their original height after washing in saline.

β-radiation affects p.d. and irritability in the same direction. The end-plate-fibre potential decreases. If the radiation is continued the resting potential of the fibre is also abolished and, simultaneously with structural changes, the preparation loses its indirect and direct excitability. If the exposure is interrupted in time the effect of the β-rays is reversible, both p.d. and indirect irritability being restored.

In order to measure the changes of the end-plate-fibre potential under excitation and its relation to the subsequent electrical phenomena a special electrostatic balanced D.C. amplifier with cathode-ray oscillograph was used (Buchthal and Nielsen 1936). The stimulus was a single cycle pulse of symmetrical shape, generated by swinging an electromagnet past a stationary coil (Buchthal 1937*b*). The same electrodes were used for stimulation and for measuring the potential. The stimulus escape was avoided by a switching device which permitted the amplifier and preparation to be disconnected during stimulation and to be reconnected after 1 msec.

Stimulation of a single fibre under isometric conditions results in two different electrical phenomena, a rapid change and a slow one, the former probably related to the excitatory change in the transmitting system, the latter to the changes accompanying and following contraction. Both potential changes are diminutions of the two resting potentials (end-plate-fibre and fibre-fibre respectively).

This observation is possible only in lizard's muscle fibre where the end-plate is localized. In the indirectly stimulated frog's muscle fibre a similar curve is obtained, but the relationship of the first transient with the resting potential of the transmitting system cannot be established since the nerve endings are spread over a wide area. It is possible, however, to imitate the conditions of the frog's muscle fibre in the fibre of the lizard muscle by placing both electrodes on the fibre and stimulating the end-plate by current escape. There is therefore no essential difference between the electrical reactions of the two fibre types.

On the completely *curarized fibre*, where the potential difference between end-plate and fibre has disappeared, direct stimulation is not accompanied by two separate changes but by a single abrupt decrease of the fibre's resting potential.

The occurrence of potential changes during excitation and contraction does not exclude the presence of a chemical mediator, liberation and disappearance of which may be correlated with the electrical phenomena.

G. L. Brown—*Neuromuscular and Ganglionic Transmission by Acetylcholine*

The full evidence which has led to the conception of chemical transmission in processes involving junctional delays of the order of fractions of a millisecond can only be summarized here, but more complete accounts are available in two recent reviews (Eccles 1936*b*; Brown 1937*d*). In brief, it may be stated that acetylcholine is liberated in the innervated tissues by impulses in the nerve whose effects are to be transmitted. (Feldberg and Gaddum 1934, ganglion; Dale, Feldberg and Vogt 1936, skeletal muscle.) This acetylcholine is liberated from the terminals of the preganglionic nerve (Brown and Feldberg 1936*a*; Feldberg and Vartiainen 1934) and the motor nerve (Dale, Feldberg and Vogt 1936). The innervated tissues are sensitive to acetylcholine applied to them through their circulation; the ganglion discharges impulses along its post-ganglionic fibres, and the muscle responds with a series of contractions, arising as normally propagated waves from the motor end-plate in the mammal, and from the region of the nerve ending in the frog (Brown 1937*a*, *b*, *c*). Acetylcholine acts directly upon the cell and not indirectly through terminal nerve filaments, as shown by the persistence of the response to acetylcholine of ganglion cells and muscles, after degeneration of their pre-ganglionic and motor nerves (Brown 1937 *b*, *c*). Such a direct excitation of the muscle fibre by acetylcholine renders less likely the supposition that acetylcholine has only a subsidiary depolarizing action in neuromuscular transmission (Monnier 1936).

If acetylcholine is to act as the transmitter in these tissues, it must be liberated within the synaptic or neuromuscular transmission time, and must disappear almost equally rapidly, viz. within the refractory period of the responding tissue. To account for this necessarily rapid destruction a high concentration of the specific hydrolytic enzyme, cholinesterase, has been postulated as existing either in the nerve terminals or closely associated with them. Direct evidence for such a concentration of enzyme in the ganglion has been supplied by the experiments of v. Brücke (1937), and for the nerve endings of muscle by Marnay and Nachmansohn (1937 *a*, *b*), and Marnay, Minz and Nachmansohn (1937). If the rapid removal of acetylcholine is, in fact, to be attributed to the action of esterase, inhibition of the enzyme by eserine or by one of its analogues might be expected profoundly to influence the transmission process. The evidence on the action of eserine on ganglionic transmission is conflicting (Eccles 1936*b*), but in the case of mammalian and amphibian skeletal muscle, the effects of inhibitors of esterase are much more clear cut (Brown, Dale and Feldberg 1936; Brown 1937*a*; Feng 1937). The administration of an inhibitor of esterase, under suitable conditions, converts the muscular response to a single nerve volley from a single contraction to a tetanus of some duration, and there is a direct relationship between the antiesterase activity of a substance, determined *in vitro*, and its capacity for producing this effect (Bacq and Brown 1937).

There is, thus, a very close similarity between the evidence for chemical transmission at the peripheral endings of the autonomic system, and for a similar intervention at the ganglionic synapse and voluntary neuromuscular junction, but the analogy cannot be extended to the details of the transmission process. In peripheral autonomic transmission the chemical excitant persists long after liberation, and, as a corollary of this, the effects of nerve impulses are summed peripherally by simple addition of the effects of the persisting transmitter. This long persistence has the additional effect of allowing the transmitter, by diffusion, to act on cells of the same tissue at a distance from its immediate site of liberation, and, in a few instances, to allow the diffusion of the transmitter to other tissues (e.g. the Sherrington phenomenon). These characteristics are essentially absent in ganglionic and voluntary neuromuscular transmission. All the available evidence points to a close localization of the liberated acetylcholine to its immediate site of action, and its persistence there, above a threshold intensity, for a period less than the refractory period of the responding cell. If it is caused to persist by inhibition of the local esterase, it may cause repeated discharge, as in the case of mammalian and amphibian skeletal muscle, but an accumulation beyond a certain concentration leads to disorganization of the transmitting process (Brown, Dale and Feldberg 1936; Bacq and Brown 1937; Brown 1937 *a*; Rosenblueth, Lindsley and Morrison 1936).

The effective transmission of an impulse in the sympathetic ganglion leaves behind it a condition of raised excitability of the ganglion cells (Eccles 1936 *b*), and a similar raised excitability persists in the junctional tissues of muscle after transmission (Bremer 1930). There is no reason to believe that this excitatory state is directly due to the persistence of the transmitter. The persisting excitatory state can be set up by antidromic excitation of ganglion cells (Eccles 1936 *b*) and probably by direct excitation of the cells of the central nervous system by an induction shock (Lorente de Nó 1935). The chemical transmitter in the ganglion and muscle, therefore, must be regarded as exciting momentarily and disappearing, and persisting changes in cellular excitability must be properties of the cell, whether it is set into activity by the normal transmitter or by some artificial means.

Many of the phenomena of neuromuscular transmission demand reinvestigation in the light of the theory of chemical intervention, and *a priori* assumptions must be made with caution. The existence of an "all-or-nothing" impulse in the nerve fibre, and a similar response arising from the ganglion cell and muscle end-plate, does not necessarily imply that the amount of acetylcholine liberated by each impulse obeys the same law. In fact, there is reason to believe that the first of a series of volleys in a motor nerve may liberate a greatly supraliminal charge of acetylcholine, and that subsequent volleys may discharge less in proportion to the frequency at which they succeed one another (Brown 1937 *d*). On this assumption, the transmission process must be regarded as involving the interplay between a transmitter liberated in varying amounts and a receiving cell whose threshold can vary, apart from external influences, according to the persisting changes in excitability produced in it by previous excitations.

A. M. MONNIER (*Sorbonne*)—*Present State of the Electrical Theory of Transmission*

At the present time, while transmission of excitation along a continuous structure like a nerve fibre is still generally considered as following the local electrical circuit theory, transmission between adjacent structures has been demonstrated in many cases to depend upon the liberation of a chemical mediator. But the exclusive generalization of this theory is prevented by the fact that some drugs are able considerably to decrease the sensitivity of the effector to the mediator while leaving neuromuscular transmission unimpaired (action of atropine on the bladder, of 933 F upon the nictitating membrane). Consequently it has been suggested (Monnier and Bacq 1935; Eccles 1936*b*) that neuromuscular transmission of excitation may normally depend upon two coexisting mechanisms, a chemical one and a physical one, which may either predominate according to the type of muscle considered. Recent experiments of Tournade and Chevillot (1937) show that not only excitatory processes, as was first suggested (Monnier and Bacq 1935), but also inhibitory actions may be elicited in the effector by the nerve impulse without the apparent intervention of the usual mediator. The kidney of a dog I is irrigated through the cervical vessels of a dog P. The kidney is connected to a plethysmograph. Vaso-dilatation of this kidney is thus recorded. It can be elicited either reflexly through stimulation of Hering's nerve of dog I or through injection of acetylcholine in the renal artery of the same dog.

But if atropine is injected in the blood of dog P stimulation of the Hering's nerve of dog I produces as before reflex vaso-dilatation in the kidney of the same dog, but the action of acetylcholine is *reversed*: acetylcholine elicits the vaso-constriction of the kidney.

After ergotamine in renal artery I, acetylcholine injected in the same artery produces again its normal effect, i.e. vaso-dilatation in kidney I. But stimulation of Hering's nerve fails henceforth to produce any vaso-dilatation reflex. It appears therefore justified to reconsider that, for example, transmission of excitation between a nerve fibre, and the corresponding terminal structure may be attributed to the electrical stimulation of the latter by nerve action potential as was postulated by the electrical theory. Such theory is not a fiction, as direct evidence of electrical transmission between contiguous cells has been given by Osterhout and Hill (1930) (salt bridge experiment). Synchronization of rhythmical impulses in non-myelinated nerve trunks appears to depend upon the mutual electrical excitatory processes produced among the fibres by their action potentials. The electrical excitatory process exerted by the nerve action potential are to be inferred from the results of direct electrical stimulation of various tissues. From these results Lapicque (1926) has formulated the conditions which, when fulfilled, ensure easiest electrical transmission between two structures, as for instance between nerve and muscle: (1) A definite ratio between the duration of the nerve action potential and the time factor of electrical excitation (chronaxie) of the muscle, a condition which is equivalent to that obtained when nerve and muscle chronaxies approach the same figure. (2) A certain ratio between the magnitude of the nerve action potential and the intensity factor (rheobase) muscle excitation.

However, recent work has seemed at first to invalidate the experimental basis of the above conclusions. Rushton (1931) has clearly shown that the skeletal muscle may exhibit a strength-duration curve with such a large time scale that it apparently excludes the first of the conditions formulated by Lapicque (1926), i.e. equality of nerve and muscle chronaxies. Rushton's results are essentially to be attributed to his method of muscle stimulation (i.e. stimulation exerted on a large area of the muscle). Indeed, further investigations (Lapicque 1932; Benoit 1934) have confirmed older observation (Davis, Watts), namely, that apparently muscle chronaxie increases with electrode surface. Only muscle chronaxies determined with small electrodes are related to the functional rapidity (Lapicque) and vary with temperature according to a Q_{10} similar to that of other physiological processes (Benoit 1934). These chronaxies, which besides are close to that of the corresponding nerve, are physiologically significant.

Benoit (Benoit, P. H. and M. 1936) has also demonstrated that the responses elicited on skeletal muscle with large electrodes are not a single twitch but are rhythmical. Rhythmical response of muscle depends upon a large stimulating cathode, just as is to be observed on Lillie's iron-wire model and on various tissues (*Nitella*) (Auger 1935).

Transmission of excitation, by such an electrical stimulus as the nerve action potential, can be shown to depend upon the sensitivity of the muscle to slowly rising current, sensitivity which is defined by von Kries and Keith Lucas "minimal gradient".

Mathematical treatment of the transmission problem, supported by physical analogies (Monnier 1934), shows that transmission results from a phenomenon closely resembling resonance (para-resonance). Bluntness of para-resonance curves depends upon the ratio of the time constants of the two exponentials τ_2, τ_1 which ratio is assumed to depict the "excitation state" elicited by a constant current. This ratio, though larger for skeletal muscle than for nerve, appears not to vary widely in normal tissues.

A. V. Hill (1936) has proposed a similar mathematical treatment with the addition that the ratio of the above time constants is subject to wide variations according to the conditions of the tissue. Indeed, this author and Solandt (1936) have published experiments in which this ratio is so large as to give to the para-resonance curve such a bluntness that para-resonance appears no more as a condition for transmission. But as a large ratio of the time constants interprets also a small "minimal gradient", it can be shown by experiments with exponentially rising currents that small "minimal gradients" (i.e. large ratio of time constants) are to be attributed to rhythmical responses.

On skeletal muscle small "minimal gradients" can be obtained only with large electrodes (which precisely are necessary for rhythmical response) and are best to be observed on decalcified muscle (Benoit and Coppée 1935). The same results are to be obtained on nerve but only when the nerve is either excised from a frog kept for several days at low temperature ("Kaltfrosch") or treated with a decalcifying agent (sodium citrate or oxalate) (Benoit, P. H. and M. 1937). When the time constant of a current exponentially rising is progressively increased, the action potential of a normal nerve diminishes and eventually disappears. This decrease of amplitude is not to be attributed to a spreading of the action potentials of the various nerve fibres, but results from a gradual reduction of the number of active fibres. The same result is obtained on a decalcified or "Kaltfrosch" nerve. But in that case, prolongation of the current elicits a rhythmic response. This response, even if the current rises rapidly, frequently occurs after a considerable delay. The time constant of the exponentially rising current which cancels out the normal nerve action potential or the first action potential of a nerve showing rhythmic responses is important (Lapicque 1937). It is correlated to the chronaxie. Mathematical analysis shows that these results depend upon a small ratio of the above time constants τ_2, τ_1. Consequently transmission may be based upon a para-resonance phenomenon. Furthermore, Fessard's (1936*a*) theory of a dual process of excitation leading to two modes of response (single and repeated) receives much support.

J. C. ECCLES—*The Responses which Nerve Impulses evoke in Nerve and Muscle Cells*

An impulse in any normal nerve fibre eventually reaches a junctional region where this fibre establishes functional connection with an effector cell, e.g. with a neurone, a muscle fibre, etc., such an impulse only achieving physiological significance by the action it exerts on the effector cell. Such actions have been investigated in relation to the functional connections between nerve fibres and neurones (the synapse) or muscle cells (the neuromuscular junction), and they appear to be divisible into the following two categories (cf. Eccles 1937).

1—The prolonged action (seconds or minutes) of a chemical transmitter which nerve impulses cause to be secreted at the junctional region. During this action the rhythmic production of impulses by the effector cell may be depressed (vagus on heart) or increased (accelerantes on heart), the rhythmic production of impulses even being initiated in a previously quiescent effector cell (action of sympathetic on smooth muscle of cat's nictitating membrane, Eccles and Magladery 1937). With some effector cells these excitatory changes have been shown to be associated with a change in demarcation potential, and hence presumably in surface polarization. Thus, both with the heart and with smooth muscle, a decreased polarization is produced by an excitatory transmitter, and an increased polarization by an inhibitory transmitter (Gaskell 1887; Bacq and Monnier 1935). Since a corresponding association is observed respectively at the cathode and anode of a constant current (Reinmüller 1932; Erlanger and Blair 1936; Fessard 1936*b*; Katz 1936*b*), it is generally agreed that polarization changes are at least partly responsible for the actions which chemical transmitters have on effector cells.

The prolonged action of a high concentration of an excitatory chemical transmitter may depress the excitability of the effector cell, e.g. the block in neuromuscular transmission and the accompanying contracture which rapid tetanic stimulation sets up in amphibian striated muscle (Feng 1936; Feng and Shen 1937) and in the smooth muscle of the nictitating membrane (Eccles and Magladery 1937), and the synaptic block on rapid tetanic stimulation of a perfused sympathetic ganglion (Brown and Feldberg 1936*b*). This condition is probably due to the further depolarization of the effector cells, for Cowan (1936) finds that acetylcholine produces very large depolarizations of frog's sartorius under conditions when it would undoubtedly be giving contracture responses (cf. Brown 1937*b*).

All these prolonged actions of the secreted chemical transmitters are closely paralleled when by injection they are applied directly to the exteriors of the effector cells.

2—The actions of a transmitter which is clearly distinguished from the above by its extreme brevity (at most a few milliseconds), and which may be either the action current of the impulse or a rapidly destroyed chemical substance. These actions are as follows:

(*a*) When a nerve impulse enters into functional relationship with a nerve cell across a synaptic junction, it produces in that cell a very brief excitatory condition which has been called a detonator response (Eccles 1936*b*, p. 358) because it appears to be directly responsible for setting up the discharge of an impulse by that cell—essentially an explosive act. The detonator response is not associated with any detectable electrical potential, and it bears a close resemblance to the local excitatory state of peripheral nerve. Nerve impulses also probably set up detonator responses in smooth and striated muscle, but not in cardiac muscle.

(*b*) If the detonator response attains a certain critical intensity, an impulse is generated in the effector cell and is propagated over the whole of that cell. In every respect this impulse seems to be identical with an impulse set up in the effector cell by direct stimulation or secondary to an antidromic impulse.

(*c*) Following the detonator response of a nerve cell there is a more prolonged phase (about 150 msec. with ganglion cells) of increased excitability, the central excitatory state (c.e.s.), which is associated with a negativity of the soma relative to the axon (Eccles 1935), i.e. presumably with a partial depolarization of the soma. This relatively long duration of c.e.s. is not due to a prolonged action of a synaptic transmitter, for c.e.s. of a similar duration is produced by an antidromic impulse (Eccles 1936*a*). With ganglion cells, c.e.s. appears never to reach a sufficient intensity to set up the discharge of impulses, but with motor neurones a discharge may occur even during the c.e.s. set up by an antidromic volley (Eccles and Pritchard 1937). Since c.e.s. is produced in effector cells even when no impulse is set up in them, it is not closely analogous with the supernormal phase and associated negative after-potential of peripheral nerve. Prolonged excitatory states, presumably analogous to c.e.s., are also produced by nerve impulses acting on the neuromuscular junctions of striated (Bremer 1927) and smooth (Eccles and Magladery 1937) muscle, in this latter case being associated with a local depolarization.

(*d*) The discharge of impulses from nerve cells secondarily produces a prolonged subnormal phase of excitability (the central inhibitory state or c.i.s.) and an associated positivity of the soma relative to the axon (Eccles 1935, 1936*a*; Eccles and Pritchard 1937). This c.i.s. appears to be a special development of the subnormal state and associated after-positivity of peripheral nerve. C.e.s. and c.i.s. are independent responses reciprocally related to one another, so that, when excess of either is present, the nerve cell reacts in the opposite direction (Eccles 1935, 1936*a*).

The special responses which are evoked in effector cells by nerve impulses acting at junctional regions thus appear to be special developments of reactions which can be produced in those parts of the effector cells which have been differentiated for another purpose, e.g. in the axon of a nerve cell. Where no close resemblance is demonstrable, e.g. with c.e.s., it may be that this special development of the junctional region has caused it to deviate considerably from the presumably undifferentiated response of the axon—in this case the supernormal phase.

W. A. H. RUSHTON—*Some Factors Involved in the Initiation of the Nervous Impulse*

Hodgkin has shown that when a nerve is conducting an impulse, the electrical energy liberated by the active region is easily sufficient to excite the neighbouring inactive stretch, and hence will account for the mechanism of propagation. But the energy arising from a fully established impulse is due to the activity of a nerve stretch many millimetres in length and will hence be far greater than that arising from a very small active region such as will occur round the cathode of a stimulating circuit. In fact, unless the stimulus raises to activity a certain definite length of nerve, propagation will not take place.

Now the classical considerations with regard to the process of excitation suppose that the applied current charges or depolarizes some element in the tissue, and when this process has reached a critical level, a sudden liberation of energy occurs, which causes propagation. If we accept this, it follows that we must consider a threshold excitation to occur, not when a *point* has been raised to activity; but when this condition has been established over a length of nerve sufficient to liberate the energy necessary to propagate.

The exact mathematical treatment of this question is very difficult, and I shall give here the results of an analysis in which the following over-simplified assumptions are made. The nerve is assumed to be a non-capacitative cable, the current passing through whose sheath also charges a leaky membrane of resistance negligible in comparison. When any element of the membrane attains some critical charge, we suppose that it suddenly liberates its stored energy, and that this causes the potential difference across the membrane to change suddenly and to persist indefinitely.

From these assumptions it can be shown that the necessary and sufficient condition for propagation is that a certain fixed length of nerve should be active, at the moment when the stimulus ceases. If this liminal length has not all been excited, the activity will remain localized.

If, then, we are given a stimulus of any time-course whatever, applied to the tissue in any given space distribution whatever, it is possible to calculate with only two arbitrary constants, the excitation produced. The results of these calculations coincide with the known experimental results over a considerable range, and in this respect are not inferior to the conclusions of other theories with more restricted scope. The more interesting results, however, are those deductions which differ from the expectations of the classical theories. One such is in excitation by a constant current.

Classically we expect that, as the charge accumulates, so the rate of removal will increase, so that the rate of accumulation of charge will diminish continually from the onset of the current until the moment at which excitation occurs. On the present theory, this state of affairs holds only up to the moment at which the point under the cathode becomes active. From this time onwards, the applied constant current is augmented by the action current of the growing excited region, and this causes the rate of accumulation of charge to increase continually up to the point at which the whole liminal length is active, whereupon propagation will continue even if the applied stimulus is withdrawn.

What does in fact occur may be found by superposing a brief shock upon the constant current at various times after the start, and at the same instant cutting off the constant current. The threshold strength of shock at each instant gives the amount of charge still required to complete excitation. Investigated in this way (Rushton 1932), it was found that excitation increases in accordance rather with the present theory than with the classical ones.

Another deduction from the "liminal length" is that if the cathode lies between two anodes so close together that the cathodal stretch is less than the liminal length, conduction will never occur, whatever the intensity of the stimulus. This is the state of affairs when a single muscle fibre is excited through a closely applied pore electrode, where only a localized effect occurs, without propagation (Pratt 1930; Gelfan 1930).

The main value of the present theory, however, lies in its attempt to embrace in an approximately quantitative fashion the whole field of excitation, and to indicate the relations between this and the quantitative data from conduction measurements. It contains many suggestions for experimental criticism, and, in the light of these, we may expect to obtain definite information as to the directions in which the present tentative ideas should be developed.

B. H. C. MATTHEWS—*Do the Rhythmic Discharges of Sense Organs and of Motor Neurones Originate in the Same Way?*

In recent years many authors have shown that long-lasting potentials are associated with the discharge of impulses in ganglionic systems (Adrian 1931; Eccles 1935; etc.). Some analysis of the occurrence of such potentials in the vertebrate spinal cord has also been made (Gasser and Graham 1933; Gasser and Hughes 1934; Umrath and Umrath 1934; Barron and Matthews 1936; Eccles 1937 *b*; Hughes, McCouch and Stewart 1937).

Barron and Matthews have found that the discharge of motor neurones evoked by sensory stimulation is always preceded by a central depolarization of the motor fibres which spreads along the motor roots electrotonically, where it can be observed and recorded. This is illustrated in fig. 5, showing depolarization preceding and accompanying the impulse discharge of a flexor reflex recorded from a small ventral root twig in a cat and a frog. The impulse discharge ceases when the depolarization ceases; the depolarization may persist for minutes with prolonged sensory stimulation. The depolarization appears to be closely associated with the central state causing the motor neurones to discharge, and when single units are observed the frequency of response varies with the magnitude of the depolarization. Artificial depolarization can be applied by placing an anode on a ventral root and a cathode on the cord; this evokes a rhythmic discharge from the motor neurones with currents of the order one microampere per square millimetre of anterior root. The response of single units can be observed by placing recording electrodes on the ventral root distal to the anode and subdividing the nerve. The response in single units increases with the current from 0 up to 40–70/sec. This is illustrated in fig. 6. With steady currents the response continues for long periods with little change in frequency. Controls show that the current is taking effect on some structure peculiar to the motor neurone, and not on the fibres under the electrodes. Direct current stimulation and reflex sensory stimulation are additive, and current in the reverse sense hinders reflex discharge.

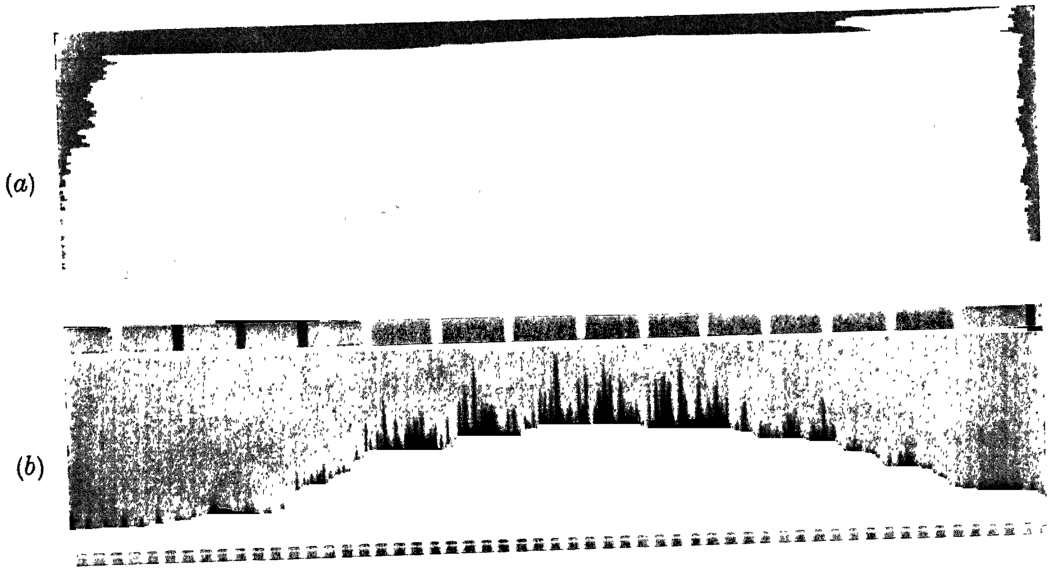

FIG. 5—Potential changes in anterior root during flexion reflex evoked by pinching the ipselateral foot in spinal animals. Direct coupled amplifier. (*a*) frog, electrodes 3 and 9 mm. from cord. 150 microvolts = 1 cm. (*b*) cat, electrodes 2 and 10 mm. from cord. 100 microvolts = 1 cm. Upward deflexion indicates proximal lead negative to distal. Time marker, 1/10th sec. intervals, A; 1/50th sec. intervals, B.

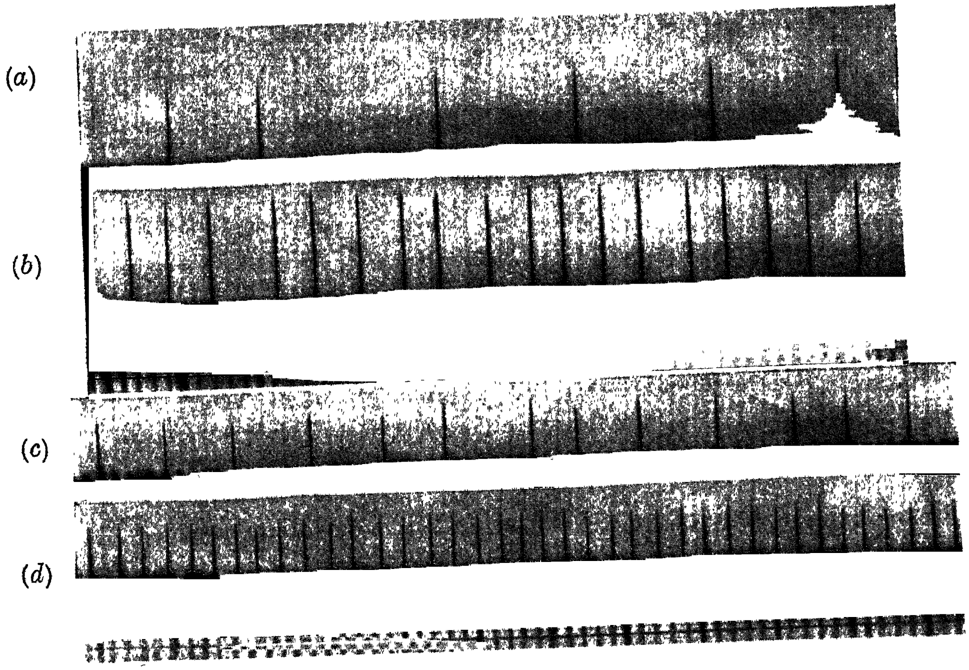

FIG. 6—Rhythmic discharge of single motor neurones evoked by stimulation with direct current. (*a*), (*b*), cat, polarizing current 1 and 2μA. (*c*), (*d*), frog, polarizing current 0·5 and 0·75μA. Time 1/10th and 1/50th sec. intervals.

It is suggested that the motor neurone may be regarded as a structure having the same properties as a sensory nerve ending, and responding to the physico-chemical state of its immediate neighbourhood, and is a "sense organ" to central excitatory state. This view is supported by the observation that simultaneous antidromic stimulation 25 times/sec. does not materially alter the changes shown in fig. 5 (except of course to reset the units), from this the prolonged depolarization is thought to be due to changes external to the motor neurone stimulating it as does a constant current. In the sensory nerve ending indirect evidence suggests that the stimulus acts by producing a depolarization to which the ending responds rhythmically, the only direct evidence that such a depolarization occurs is that of Hartline (1932) on visual sense cells. Sensory nerve endings also set up a rhythmic response to direct current stimulation (Fessard, unpublished).

The table below sets out parallel properties of motor neurones and sensory nerve endings; as Adrian and Bronk (1928) have suggested, similarities may result from the common properties of polarized surfaces. Much support is lent to this view by the recent work of Fessard (1936*b*) on rhythmicity in isolated nerves.

Motor neurone	Sensory nerve ending
Steady (reflex) excitation yields rhythmic response (Adrian and Bronk 1928)	Steady excitation yields rhythmic response (Adrian and Zotterman 1926)
Rate of response rises with increasing excitation (Adrian and Bronk 1928)	Ditto (Adrian and Zotterman 1926)
Antidromic impulses reset rhythm with compensatory pause (Eccles and Hoff 1932)	Ditto (Matthews 1933)
Rhythmic response to deformation (Adrian and Bronk 1928)	Ditto
Natural reflex excitation produces depolarization, rate of rhythmic response increases with depolarization (Barron and Matthews 1936)	Depolarization precedes response to natural stimulation? (Visual sense cells, Hartline 1932)
Rhythmic response to steady current excitation. Frequency increases with current (Barron and Matthews 1936)	Rhythmic response to steady current excitation. Frequency increases with current (Fessard unpublished)

Z. M. Bacq (*Liége*)—*Cholinergic Nerves in Invertebrates*

The presence of acetylcholine (ACh.) and choline esterase has been demonstrated in the tissues of the molluscs, worms and echinoderms. The muscles of these animals are sensitive to ACh. (Bacq 1935). Their position is thus very similar to that of the vertebrates. Coelenterates and crustaceans have no acetylcholine; their muscles are unsensitive to ACh.; furthermore, coelenterates are without choline esterase. In these two classes of invertebrates, the existence of cholinergic nerves is impossible to conceive. Tunicates have little choline esterase in the muscles; the presence of ACh. is not demonstrable; the concentration required to cause contraction of Ciona's muscle is high. Thus, in tunicates, the existence of cholinergic nerves is unlikely.

So far, our attention has been concentrated on worms and molluscs. In collaboration with G. Coppée (Bacq and Coppée 1937), I have shown that eserine in concentrations from 1/200,000 to 1/20,000 appreciably increases, sometimes tenfold, the tension developed by various worm muscles in response to maximal shocks to the nerve. We used longitudinal strips of *Sipunculus nudus*, *Hirudo medicinalis*, *Lumbricus terrestris*, *Arenicola marina*, *Aphrodita aculeata*. We have seen, in the eserinized leech, the effect of a single stimulus last for 15 min.

When a piece of ventral muscle of the leech is stimulated through the nerve, a substance diffuses into the surrounding fluid which has all the properties of an unstable choline ester: it is destroyed in absence of eserine; its contracting action on the denervated leech preparation is increased by eserine; its depressor action on the cat's blood pressure is abolished by atropine.

Thus our conclusion is that the motor nerves of the worms are cholinergic.

Not a single neuromuscular or synaptic junction of a mollusc is known to be affected by eserine. Kruta (1936) has investigated the action of visceral inhibitory nerves on the median ventricle of cephalopodes; personally, I have tried on the same animals the perfused preparation of the mantle. Eserine and the more powerful esterase inhibitor methylurethane of *m*-hydroxyphenyltrimethyl ammonium iodide are without action on the stellate ganglion of *Eledone*, and acetylcholine, even after eserinization, has no action on the nerve cells of this ganglion (Bacq 1937).

With Coppée, I have recently observed that immersion during 1 hr. in eserine 1×10^{-4} does not alter the response of two mollusc muscles to quantitative nerve stimulation (foot of *Buccinum undatum*; siphons of *Mya arenaria*).

Does this mean that motor nerves of Molluscs are not cholinergic? Very likely, since other facts corroborate the absence of action of eserine:

(1) Excitation of nerves does not increase the ACh. content of eserinized perfusates in Cephalopodes (Bacq 1937).

(2) Degeneration of preganglionic fibres in the mantle nerve does not

result in the disappearance of the ACh. content of the stellate ganglion (Bacq 1937).

It seems that, in molluscs, the nerves cannot mobilize the ACh. available in the innervated tissues. And we fall back again upon the much debated alternative: the transmission is chemical, the mediator not being a choline ester; the transmission is physical, the eddy currents at the nerve ending acting as direct stimuli to the muscle fibre.

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Quantitative Spectrographic Analysis of Biological Material—II

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[Plate 16]

Introduction

It is well known that several metallic elements in small concentrations are essential to plant growth and normal development. The relative importance of a given element and the concentrations necessary or most beneficial vary with the plant. Quantitative spectrographic methods permit one to determine the distribution of the element throughout a single plant and in certain cases follow the distributions through the life of an individual specimen. Such methods may therefore be expected to aid in the solution of many problems now fully appreciated by biologists and to suggest further studies.

For many years it has been known that boron deficiency has disastrous effects on many plants of economic value such as sugar beets, tomatoes, turnips, tobacco, and celery. Chemical analysis as applied to studies of remedial treatments or of a purely scientific nature is relatively long, requires much material, is less sensitive, and is such as to risk loss of boron before the analysis is completed.

The present development of suitable spectrographic analysis of plants for boron has been made with the following aims: (1) use of small samples, (2) minimum preliminary treatment, (3) reproducibility, and (4) speed. It has been found that 200 mg. of fresh material is ample for quantitative analysis. There is no ashing or chemical treatment. Results rarely vary as much as 10% from the mean in a long series, and the time required per analysis is about 1 hr. There is a somewhat greater sensitivity than is required in practice.

It was first established as a fact that when a sample containing boron is excited in a suitable spark discharge, the intensities of the sensitive boron lines 2496 and 2497 A are proportional to the number of atoms present over the range of concentrations normally found to be of practical interest (fig. 1). As an internal standard, gold is introduced into duplicate samples. After the further addition of a known amount of boron to one of them, the two samples are sparked. From the relative intensities of the boron to gold lines in the two spectra, the amount of boron originally present in the sample is determined.

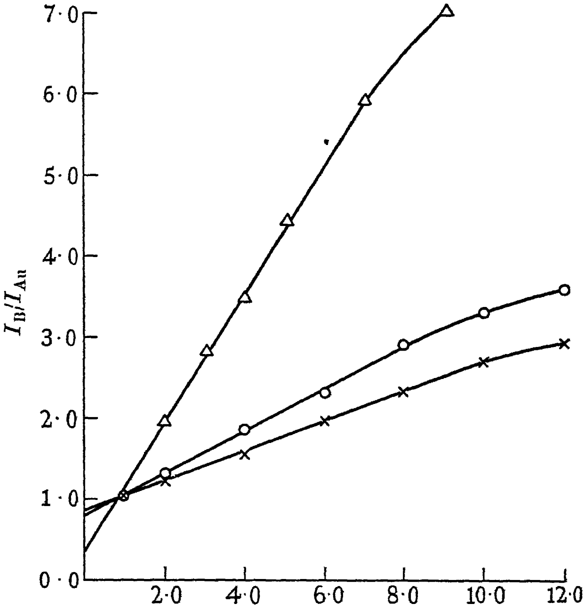


FIG. 1—Concentration of added boron × 10^6 g./c.c.

In the application of this method to plant tissue some modifications of technique are required to deal with certain features not met in the analysis of fluids (Foster, Langstroth and McRae 1935). Since there are variations in the distribution of the element over small regions, the weighed sample from a fresh plant is made representative of the material to be investigated, yet not too bulky on the electrode. The internal standard and boron solution are thoroughly mixed with the sample, crushed and disintegrated in a small copper mortar. Before the sample is spread on a small plane electrode, a binding material is added so that the sample dries in a thin compact layer in good contact with the metal. This is necessary, since otherwise the explosive force of the first few sparks would cause too great loss of material. By this treatment one also prevents the appearance of loose flakes of the sample which would mask one section of the spark and thus cause a change in the relative intensities of the lines under investigation.

EXPERIMENTAL PROCEDURE

Before starting a quantitative analysis of a new biological material for a given element, an exposure must be taken to determine qualitatively what other elements are present. The most suitable persistent spectral line of the element in question is then chosen with reference to its position in the spectrum and to possible interference from lines due to the air or to other elements in the sample. The internal standard is then selected subject to the same conditions, and the additional requirements (1) that it be not present in variable amounts in the sample, (2) that plate sensitivity does not vary appreciably toward the lines under comparison, and (3) that the lines arise from transitions between similar atomic states so that small differences in the excitation should not change appreciably the ratio of the intensities. The boron line ${}^2P_{\frac{3}{2}}$–${}^2S_{\frac{1}{2}}$ (λ 2497·7) and the gold line ${}^2P_{\frac{3}{2}}$–${}^2S_{\frac{1}{2}}$ (λ 2427·9) have been found to satisfy these conditions.

At the beginning of an analysis two samples of 100 mg. each are conveniently weighed out on a torsion balance. A duralumin arm is balanced at the centre of a violin E string 8 in. long and carries a mounting for a cover-glass to receive the sample. The material is ground to a fine pulp in the copper mortars shown in fig. 2, Plate 16. Copper was chosen as the material for the mortars due to its freedom from boron and ease of cleaning. By means of a capillary pipette graduated to deliver 0·040 c.c. of solution, 4×10^{-6} g. of gold from a gold chloride solution is added to each sample as an internal standard. In the same solution enough lithium tartrate (as binder) is included to give 2 mg. of this salt per sample. With a second pipette 0·050 c.c. of sodium tetraborate solution, giving 5×10^{-7} g. of boron, is added to one mixture. These solutions are thoroughly mixed with the pulp, and then poured out one on each electrode. If the solutions are thoroughly mixed through the pulp, no error arises from small but variable amounts of the sample adhering to the inside of the mortar.

The electrodes are rectangular aluminium plates $2·5 \times 1·3 \times 0·1$ cm. The load is placed in the central portion of the plate (approximately 1·7 cm.^{2}) marked out by a pointed piece of paraffin to keep the liquid from spreading. Before they are used, electrodes and all glassware are cleaned with chromic acid, hot nitric acid, and distilled water. Tests failed to reveal any boron in the gold or lithium solutions, in the paraffin, or in the aluminium.

The electrodes are put in a desiccator and allowed to dry slowly under reduced pressure provided by a Harrington-Cenco aspirator. If the pressure is taken down to the boiling-point, the sample does not dry in a coherent layer but tends to form a hollow crust. Boron will be lost if the drying proceeds at much higher temperatures. For example, samples dried at 80° C. by means of electrical heating when compared with like samples dried slowly reveal a loss of boron up to 50 % of the amount originally present. When properly dried, each sample is a tough compact film firmly adhering to the electrode. A photograph of an electrode before and after sparking is reproduced in fig. 7, Plate 16.

The automatic source, fig. 3, Plate 16, is carefully aligned with respect to the spectrograph; for this purpose the lamp is useful. The pointed aluminium anode is set by a spacer and clamped with its tip 4 mm. above the position of the load. It may be turned back out of the way until the loaded cathode is fitted into its mounting, and then returned to position for operation. Both electrodes are insulated from the base of the apparatus, one by a section of glass rod, the other by an ebonite fitting attached to the movable platform which supports the central column. Through a worm drive the motor operates a cam designed to move the platform uniformly back and forth with a stroke equal to the width of the load. By means of a 21 : 2 reducing gear, a duplicate cam moves the platform slowly back and forth in a direction at right angles to the first motion. The short period is about 1·5 sec. Thus after the source has been in operation for 2 min., a few hundred sparks have passed uniformly over the load, and each portion may be said to have had the same probability of excitation. To avoid possible contamination, a fresh anode is used for each sample.

In most experiments no condensing lens was employed. The centre of the source was placed on the axis of the collimator and 50 cm. from the slit. By tests it was found that the slit jaws were parallel and that the slit was evenly illuminated.

The above conditions are necessary for the wedge method of photometry which is employed. Almost in contact with the slit is a wedge of aluminium film formed by evaporation on quartz. An example of the resulting spectrum taken by a Hilger E_2 quartz spectrograph is reproduced in fig. 4, Plate 16.

The high potential D.C. source has already been described (Foster and others 1935).

PHOTOMETER FOR WEDGE SPECTROGRAMS

The relative intensity of two neighbouring lines is found from the difference in the lengths of wedge penetrated, and the wedge density. The method has been well developed by Merton (Nicholson and Merton 1916) who worked in the visible region with a neutral glass wedge corrected for dispersion by a wedge of clear glass. Assuming that the wedge has the same coefficient of absorption, a, for the two radiations of intensities I_1, I_2, and that the photographic plate is equally sensitive to their actions, the intensity of radiation which under the conditions of exposure and development just affects the plate is given by

$$I_1 e^{-\frac{a}{m} l_1} = I_2 e^{-\frac{a}{m} l_2},$$

where l_1, l_2 are observed lengths of the spectral lines, and m is the magnification within the spectrograph. The relative intensity is found from

$$\log_e \frac{I_1}{I_2} = \frac{a}{m}(l_1 - l_2).$$

Following Merton, it is convenient to define D_λ, the density of the wedge at the wave-length under consideration, as $\log_{10}\frac{I_1}{I_2}$, where it is understood that I_1 penetrates the wedge to an excess length of 1 mm. Then in general

$$\frac{I_1}{I_2} = \text{antilog}_{10} D_\lambda (l_1 - l_2)/m.$$

In any application, the factors of chief interest are obviously the density of the wedge and the difference in the lengths of the lines. There is some simplification in the attention given to each factor in the present investigation.

More recently it has been found that the absorption of certain metallic films evaporated on quartz is nearly constant through the ultra-violet region. The density of an aluminium film so deposited as to form a wedge is so nearly constant from 4000 to 2000 A that corrections are normally unnecessary. Such a wedge has been supplied to us through the kindness of Professor B. O'Brien, University of Rochester, whose careful investigation of methods of manufacture and properties of aluminium wedges has drawn attention to their usefulness. As is well known, a film of transparent oxide protects the wedge and even permits washing when necessary. The density of the present wedge as determined by Professor O'Brien at 4000 A is 0·168. Thus, using this value of D_λ, the relative intensity, R, of the boron radiation to that of gold is given by

$$\log_{10} R = 0{\cdot}168(l_1 - l_2),$$

since the magnification may be taken as unity; there then remains only the measurement of $(l_1 - l_2)$. The selection of the end of a line, however, is uncertain. Merton used a fine process screen in the preparation of "half-tone" enlargements and then chose as the "end" the last dot at the top of each image. The present method is rather more direct, and places less emphasis on the density of the photographic impression at a particular point. In fact, the problem of finding the end of the line is entirely avoided.

Under the above conditions it is apparent that the light intensity is the same for each line not only at the "ends" but at all points equidistant from the "ends". It is therefore possible to use independent optical trains for the formation of adjacent images of the two lines, and then move one image until the lines appear to taper off together. The amount of this motion is a quantitative measure of $(l_1 - l_2)$.

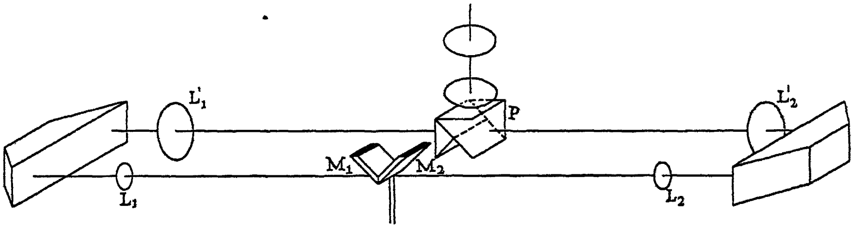


Fig. 5.

There are many ways of doing this. The first form of a photometer which is undergoing development is shown in fig. 5. Two beams of light from the evenly illuminated plate are reflected by stainless steel mirrors, M_1, M_2, and made parallel by the lenses, L_1, L_2. After passing through the trapezoid prisms, the beams are focused in a horizontal plane through the middle of the special prism, P, and the images viewed through an eyepiece. If the magnification is not the same by each path, the lenses, L_1, L_1', may be adjusted to give equal magnification. After preliminary adjustments are completed, the plate is so placed that the image of one of the lines (reflected from M_1) is in correct position for viewing, and the other mirror, M_2, together with the lens, L_2, is moved as a rigid unit until the other line is picked up. By manual adjustment the plate may now be moved slightly if necessary until the field presents the appearance of fig. 6*a*. The breaks at the bases of the lines permit an initial matching, and are made by a fine fibre across the slit near the thin end of the wedge. Finally the lens L_2' is moved by a screw until the tips of the lines match, fig. 6*b*, and the relative intensity R is read directly from a specially prepared scale on the drum. In routine work the measurements are completed very quickly. Unusual latitude is permitted in exposures, and since it is a null method, no calibration of the plate is required. Owing to a margin of sensitivity, no background appears on the plates in the region of the lines under comparison.

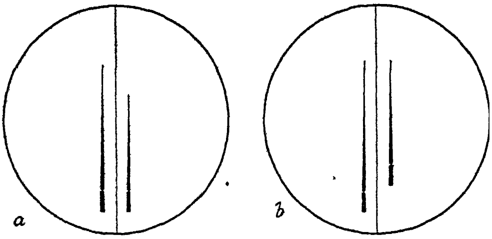


FIG. 6.

If R_1, R_2 respectively are the ratios of the boron line to the gold line in samples with and without added boron, b, then the amount of boron, B, in the original sample of mass M is given by

$$B = \frac{R_2}{R_1 - R_2} b,$$

while the amount per gram of fresh material is B/M. If the results for one plant are to be compared with those for another, it is important to weigh out samples from fresh plants each time. Following the analysis of an entire plant on the above basis, it is sometimes helpful to know the boron per gram of dry tissue in the different structures. This is easily obtained by drying independent samples, or by weighing the initial samples (and electrodes) before and after drying.

JUSTIFICATION OF PROCEDURE

The following data chosen at random from numerous tests illustrate (1) the relation of the relative intensity of the boron and gold lines to the concentration of boron, (2) the reproducibility of analysis, (3) the effect of poor grinding, (4) the effect of poor mixing of the internal standard with the pulp.

Each curve in fig. 1 is drawn from data obtained by adding known amounts of boron to like samples of material. It is found that for concentrations of boron within the range encountered in practice the relation is linear. At higher concentrations, the density of boron atoms in the spark is sufficient to cause noticeable absorption. The change in slopes and

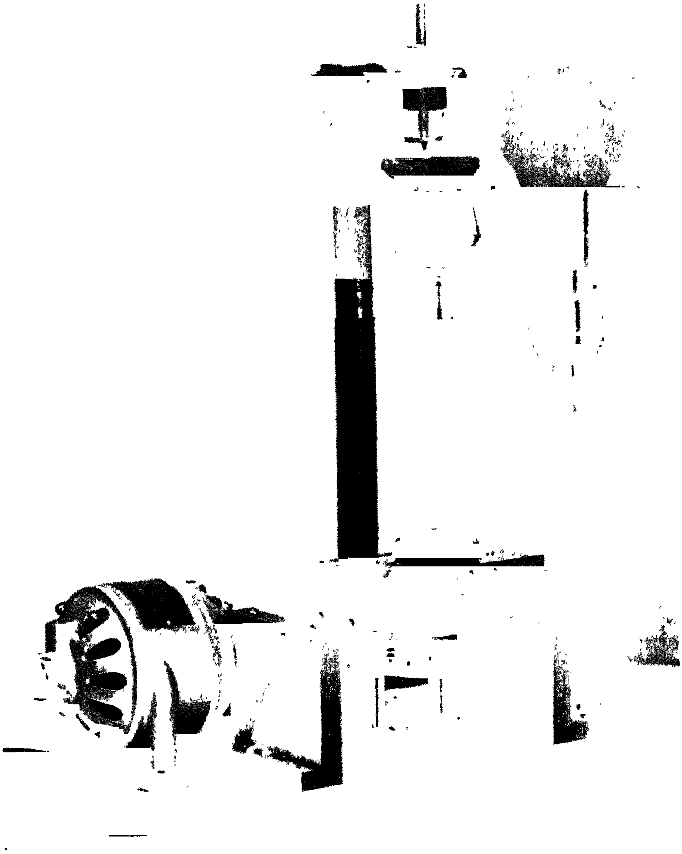


Fig. 3

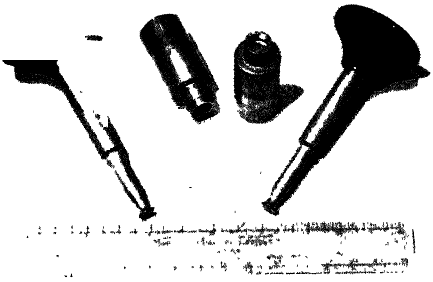


Fig 2.

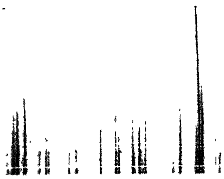
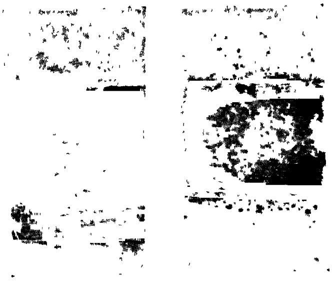


Fig. 4



a *b*

Fig. 7

(*Facing p.* 428)

intercepts from one curve to another is of course due to the use of different concentrations of gold, and to varying amounts of boron originally present in the samples. Such a calibration curve is always drawn before routine analysis for a new element is undertaken.

TABLE I—REPRODUCIBILITY OF BORON DETERMINATIONS. THE AMOUNTS OF BORON ARE MULTIPLIED BY 10^6 AND ARE IN GRAMS OF BORON PER GRAM OF GREEN TISSUE

Determination ...	1	2	3	4	5	6	Mean
Turnip leaf under standard treatment	4·2	3·9	4·2	3·7	4·3	4·1	4·1
% deviation from mean	2·4	4·9	2·4	9·9	4·9	0·0	4·0
Samples from same leaf, poorly ground	2·4	3·9	4·1		Mean boron content 4·2		
% deviation from accepted mean	41	4·9	0·0				
Like samples well ground, but gold solution added after drying	3·2	6·3	3·7	7·1			
% deviation from accepted mean	22	54	9·9	73			

The first section of Table I shows the results of six determinations made under conditions adopted as standard. From this test and many others, it may be concluded that in practice one is justified in assuming that the error in a single determination is not in excess of 10 %. It is important, however, to know what errors may be introduced by some departure from the standard conditions. These are shown in succeeding sections of the table, where the procedure has been varied in the most inadvisable manner. Some of the errors are explained by a simple consideration of one aspect of the discharge. The aluminium disk stuck on the anode, fig. 3, Plate 16, collects a surprising amount of material. The colour and other properties of the material suggest that most of it has never been excited at all, but has been driven from the electrode by the heat and mechanical action of the sparks. When the material is very poorly ground, it is not uniformly bound to the electrode, and in particular some appreciable portions of the sample are driven bodily from the source with consequent relative weakening of the boron line.

The effect of poor mixing of the internal standard with the pulp is illustrated in the last section of the table, where it will be noticed that the values on the average are high. The electrodes were loaded as usual but no gold was added. After the samples were thoroughly dry the gold solution was put on top and allowed to dry. It was observed that much of the liquid flowed round to the edges of the load and upon drying left the gold in that region which, as may be seen from fig. 7*a*, Plate 16, receives the least excitation. This has led apparently to high values for boron.

This method of analysis has also been adapted to the determination of traces of copper, aluminium and iron in raw paper pulp. Carbon cylinders free from these elements were bored axially and cut in sections. A layer of collodion was spread over the surface. The thick paper was cut in strips, weighed, wrapped around the cylinder and fastened down with collodion. The internal standard and known solutions were added and evenly distributed by capillary action. After the material had dried the cylinder was slipped on the end of a threaded shaft, so mounted on the source that it could be rotated under the pointed anode and at the same time be advanced in a direction parallel to the axis. Thus the sample was uniformly excited and the analysis from that point was carried out as in the above boron determinations.

We express our best thanks to Dr. J. M. Swaine, Director of Research, Dr. E. Archibald, Director of the Dominion Experimental Farms, and other members of the Department of Agriculture, Ottawa, for hearty co-operation. An account of co-operative studies still in progress will be given in a later paper. Our grateful thanks are further extended to the Rockefeller Foundation for the benefits of a grant to the University for the development of quantitative spectrographic methods under one of us.

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Heterosis and the Inheritance of Quantitative Characters

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In three recent papers (Ashby 1930, 1932, 1937) observations have been recorded on the physiological basis of heterosis. These data suggest no obvious genetical interpretation, though they draw attention to the need for a re-examination of the existing hypothesis. This paper considers the requirements for a theory of heterosis and discusses the bearing of heterosis on other aspects of size inheritance.

THE POLYMERIC FACTOR THEORY OF SIZE INHERITANCE

Heterosis involves quantitative characters. It is in fact usually described in plants with reference to height, for while many other qualities are affected in heterosis, increase in height is the most obvious phenomenon and the most easily measured. Any genetical theory of heterosis must therefore be consistent with a theory of the inheritance of size characters in general.

The hypothesis of size inheritance still most widely accepted dates from 1908–10, when Nilsson-Ehle and, independently, East published data on the inheritance of characters which exhibited no clearly marked segregation. The literature now abounds with examples of the inheritance of quantitative characters. Most of these examples accord in showing an F_1 population which is not more variable than its parents, and which is intermediate between them in size (except where heterosis is manifested), and an F_2 population *apparently* symmetrically distributed about a mean intermediate between those of the grandparents, and exhibiting a greater variance than either the grandparents or the F_1. Families bred from individuals from various points of the F_2 distribution differ markedly in their means and variance. This summary of a great mass of data is necessarily incomplete, but it specifies the requirements which a theory of size inheritance must satisfy.

East's theory is adequate to account for many of the results of size crosses, but it involves three assumptions which require modification in the light of modern work. These are: (i) lack of dominance among the factors determining size, (ii) an equal influence of these factors on the size character in question, and (iii) a linear relationship between the number of factors present and the size of the character upon which they operate. East pointed out that on these assumptions the distribution of size classes in an F_2 population is according to the terms of a binomial expansion, $(\frac{1}{2}+\frac{1}{2})^{2n}$, and under these conditions the distribution is symmetrical.

It is obvious that formal and simplified assumptions have to be made in any theory of size inheritance, but these particular assumptions are not acceptable for the following reasons: (i) The suggestion that size factors do not as a whole show dominance is contrary to experience, for some degree of dominance is characteristic of most genetic factors which have been isolated, including those which control size. East himself admits this (1916), though he does not suggest any corresponding modification of the theory. Moreover, the assumption was made to account for the symmetry of F_2 distributions. There was no proof of this symmetry, and indeed Fisher, Immer and Tedin (1932) have shown that significant skewness does exist in some of the apparently symmetrical distributions of size characters published by Emerson and East (1913). In other instances, where many factors operate in determining size, dominance bias may be present even though it cannot be disclosed by analysis; for the mean and the mode of a binomial distribution fall in the same class whether the distribution is skewed or not, and the distribution of the individuals in a small sample may be quite unlike the distribution of the whole population from which the sample is taken. This is illustrated by the following, suggested to the writer by Professor J. B. S. Haldane. If n factors, located on different chromosomes and exhibiting full dominance, affect a size character, the mean of the resulting F_2 is $np = 3n/4$. The variance is $npq = 3n/16$, the value of the third moment statistic, γ_1 is $-(p-q)/\sqrt{(npq)} = -2/\sqrt{(3n)}$, and the variance of γ_1 is $\sqrt{(6/M)}$, where M is the number of individuals in the population. When ten factors operate, as in fig. 1, the mean is 7·5, the variance is 1·875, and the skewness is $-0·3675$. In order to obtain a probability of 0·98 that the distribution is asymmetrical it is necessary to use about 500 individuals. It is unnecessary therefore to postulate non-dominance of size factors: even with considerable dominance the bias may be difficult to detect. (ii) The second assumption, that all factors controlling a quantitative character have an equal effect in determining its size, is not supported by analytical work on size inheritance. It is commonly found that size is governed by relatively few "principal" factors and a number of modifiers. Examples of this are to be found in the work of Punnett and Bailey (1914), Emerson (1916), Frost (1923), Lindstrom (1929), Sirks (1932), Rasmusson (1935). (iii) The third assumption, that there is a linear relationship between the number of factors and the size of the character upon which they operate, is somewhat misleading, in that it implies that

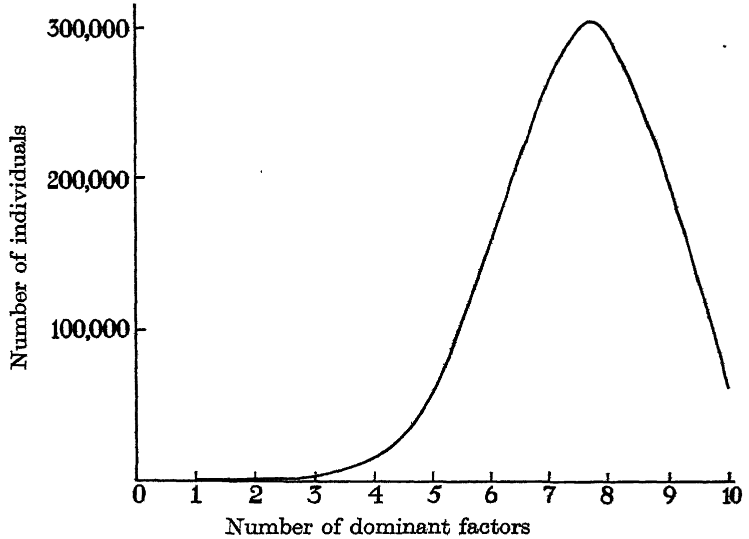

FIG. 1—The theoretical distribution of ten independently segregating dominant factors in an F_2 population. Note that in a small sample individuals with less than five dominant factors would be rare and the distribution would appear superficially to be symmetrical.

the factors are acting on a single process, whereas the size of an organ is the resultant of several more or less independent processes. Rasmusson has recently (1933) substituted for this assumption the suggestion that the effects of factors on size follow a law of "diminishing returns". This, however, carries the same implications as the assumption that factors are additive in their effect, and it may be noted that if factors acted in this way the skewness already present due to dominance bias would be greatly augmented.

THE GENETICAL HYPOTHESIS OF HETEROSIS

It is necessary to specify these difficulties inherent in East's theory of size inheritance, because this theory is at variance with the hypothesis of heterosis put forward by his co-worker, Jones. Jones's genetical interpretation of heterosis (1917) replaced the various "stimulus" hypotheses, and is still generally accepted. It explains heterosis as due to the complementary action of dominant linked factors derived from both parents. Jones's scheme provided for the occurrence of heterosis in F_1, for an assumed symmetrical distribution of size characters in F_2, and for the belief that individuals as large as those in the F_1 could not be bred true in subsequent generations. The scheme may be illustrated by the following diagram:

Parent 1			Parent 2		
AA	EE	JJ	aa	ee	jj
bb	ff	kk	BB	FF	KK
CC	GG	LL	cc	gg	ll
dd	hh	mm	DD	HH	MM

Hybrid		
Aa	Ee	Jj
bB	fF	kK
Cc	Gg	Ll
dD	hH	mM

The letters symbolize factors (in this example showing complete linkage) located in three pairs of chromosomes, and affecting the size of the organism carrying them. The factors are homozygous in the parents and they combine to give a hybrid heterozygous for them all.

On the assumptions of East's theory of size inheritance there is no heterosis according to this scheme; the "size value" of the hybrid is the same as that of its parents. Jones, on the other hand, assumes that size factors as a whole do show some dominance, and only when this assumption is made will his scheme provide even a formal analysis of heterosis.

In order to account for the apparent absence of dominance bias in the progeny of a population showing heterosis Jones postulated that the two inbred parents should make approximately equal contributions to the size of the hybrid. If this is not postulated the distribution of size in the F_2 population will be skewed, notwithstanding linkage. But the postulate is contrary to Jones's own data, for if the parents make approximately equal contributions of size factors to the hybrid, and if the size factors are equal in their potency, then *ex hypothesi* the parent plants should be about the same size for the character in question. Reference to the data on which the hypothesis is built shows that in height, for instance, the parents may differ by 40 % and there are instances of weight heterosis where the parents differ in weight by 100 % (Ashby 1930). Crossing-over, while it might reduce skewness in F_2 would not eliminate the inequality in number of dominants in the gametes if this inequality were initially large, and in the limiting case (random assortment of factors) there should still be skewness due to dominance bias. However, the assumption made by Jones is unnecessary, for there is no convincing evidence that the progenies of populations showing heterosis are *not* skewed.

It is not the purpose of this criticism to discredit the factorial hypothesis of heterosis, but to remove from the hypothesis one of those features which impair its application. Collins (1921) pointed out that Jones's attempts to account for symmetrical F_2 distributions were superfluous, in that there was no evidence that symmetry was present, but his suggestions were overlooked. In essentials, however, Jones's hypothesis is in accord with the facts. It may be restated in its modified form as follows: If two inbred strains carrying different sets of factors exerting a favourable effect on size, and showing some degree of dominance, are crossed, the hybrid may show heterosis due to the combined operation of the "favourable" factors derived from its parents. Dominance bias in the F_2 population may be detectable, or it may be concealed on account of the large number of factors contributing to it.

The hypothesis does not provide a very satisfactory basis for the analysis of heterosis, and it is significant that it has received very little direct test from experiment. Two pieces of evidence support it. Fisher, Immer and Tedin (1932) have shown, by comparing the distribution of skewness in F_3 populations with the covariance of the first and second degree statistics for the populations, that heterosis is probably due to dominance favouring the larger size. Richey (1927) and Richey and Sprague (1931) by a series of back-crosses of hybrids on their parents, have shown that the most heterozygous races show the greatest heterosis. Apart from these examples the hypothesis is justified only in that it has not been proved wrong.

A WORKING HYPOTHESIS OF SIZE INHERITANCE

Heterosis as explained by Jones is a particular instance of the operation of polymeric factors in size inheritance. A drawback to the theory of size inheritance as it stands, apart from its unsatisfactory assumptions (*vide* p. 432), and despite Rasmusson's suggested modifications, is that the theory is not helpful in indicating experiments which might carry the analysis of size further. It does not take account of the fact that factors determining size operate on different processes and at different times in the history of the organism. Analyses of F_2 distributions may be deceptive; metrical bias may obscure the bias due to dominance; and estimates, from analysis of

F_2 distributions, of the number of genes involved in size inheritance are too uncertain to carry conviction (see Rasmusson 1933; Serebrovsky 1928). A fresh statement of the theory of size inheritance, which is implicit in some modern work (e.g. Goldschmidt 1927; Ford and Huxley 1929; Sinnott 1935; Sinnott and Dunn 1935) and which emphasizes a new mode of attack on the problems, may be made in the following terms: (i) size characters are generally under the control of several genetic factors; (ii) these factors are unlikely to be arithmetically cumulative in their effect upon one single process; but the factors will operate upon various determinants of size, e.g. some upon initial size of primordia, some upon growth rates, some upon rates of differentiation, etc.; (iii) the parts played by the several genetic factors in determining size will depend upon the level of the environment and the time in the life cycle at which the factors come into operation.

The theory stated in this form indicates how the problem may be approached from another angle—by analysing size into the processes which determine it and studying the inheritance of these processes. Thus, the final size of a leaf may be resolved into the following determinants: (i) the number of cells in the primordium; (ii) the rate of cell division; (iii) the rate of cell expansion; (iv) the period over which growth is maintained; (v) the rate of differentiation; (vi) the final size of the cells. Each of these processes will be controlled by various genetic factors, and the contribution which the factors make to leaf size will be subject to the condition of the environment. The final size of the leaf is a function of so many variables that it is of little value as a measure of genotypic constitution, but the mode of inheritance of the several determinants of size may be simpler. Data on maize, for instance (Ashby 1930), show that the dry weight in the grand period of growth is referable to embryo weight and efficiency index. Two races were found to have the following values:

	Embryo weight g.	Efficiency index
Race 1	0·038	0·069
Race 2	0·013	0·097

After 43 days' growth plants from these two strains had the same mean dry weight (1·099 g.). In the one case it was reached by a small capital increasing quickly; in the other case by a larger capital increasing more slowly. The higher efficiency index was shown to behave as a complete dominant in the F_1 and the indices segregated in F_2. The segregation of embryo weights is obscured by the effect of the maternal environment, in particular the size of the testa, and it needs further analysis. This instance also draws attention to the confusing testimony of size data, for the two genotypes in question fall on different parts of a distribution for weight at different times in their development. From 0 to 43 days race 1 is heavier than race 2. At 43 days the two genotypes fall into the same weight class. From the 43rd day onwards race 2 falls into a higher class than race 1. The shape of the distribution curve depends upon the time at which the population is sampled. Such fragments of data indicate that the genetics of size may be profitably studied through a consideration of the inheritance of processes which determine size.

One unexpected fact is already emerging from these analyses: there is an indication that hereditary size differences are commonly determined by initial sizes of primordia rather than by the time relations of subsequent development. Analysis of data for maize (Jones 1917; Ashby 1932), tomatoes (Ashby 1937; Luckwill 1937), guinea-pigs (Castle, see Ashby 1937), and pigs (Roberts and Laibile, see Ashby 1937), shows that differences in size between different genotypes are referable to initial differences (i.e. at birth or germination) and are not due to differences in growth rate during post-embryonic development. Kopec's work on mammals (1923, 1926, 1933) points to the same conclusion, in that birth weight is highly correlated with subsequent size. The situation is not without exceptions, for differences in size between genotypes may depend on differences in relative growth rate (Ford 1928; Livesay 1930; Ashby 1930), but in most instances on record the ratio of sizes of adults in two strains is no greater than the ratio of sizes at birth or germination, and the genetics of size must be studied through the genetics of embryo development.

THE PHYSIOLOGY OF HETEROSIS

This method of analysis has been applied to the study of size heterosis (Ashby 1930, 1932, 1936, 1937; Luckwill 1937). The results may be summarized in the following terms: (i) heterosis in certain strains of maize and tomatoes is manifested as greater height, dry weight, leaf area, leaf number, etc.; (ii) the plants showing heterosis do not exhibit relative growth rates greater than both their parents in any of these particulars, nor have they a relatively greater respiration or assimilation rate; (iii) size heterosis is associated with larger primordia in the embryos of the F_1 population, and the additional vigour is referable in some cases entirely to this initial advantage in size. These conclusions are illustrated by the data assembled in Table I, from some unpublished experiments of Luckwill, working with the present writer. It is evident from entries 1–7 in this table that heterosis was manifested in a variety of ways. It is evident from entries 8–14 that various rates of development were not greater in the hybrid, but that the higher parental rate was inherited in the manner of a complete dominant in the F_1. Heterosis can be attributed to the initially larger primordia in the F_1 embryos, and this is in accordance with Jones's hypothesis, but the general absence of higher metabolic rates in the hybrid after germination is not what would be expected according to the hypothesis.

Table I—Summary of Data on the Degree of Heterosis and the Rates of Development in a Hybrid and its Parents. Parent 1 is *Lycopersicum racemigerum* and Parent 2 is *L. esculentum*, Recessive for Brachytic Stem, Fasciated Fruit, and "Wilty Foliage"

		Parent 1	Parent 2	Hybrid	Significance of increase in hybrid over parents
	Dry weight after 23 weeks (g.) of:				
1	Stem	90	15	130	s.*
2	Leaf	67	32	144	s.
3	Total	157	47	274	s.
4	Leaf number	284	99	319	s.
5	Mean area per leaf (cm.^{2}) ...	19·2	28·8	37·1	s.
6	Mean dry weight per leaf (g.) ...	0·129	0·183	0·246	s.
7	Embryo weight (mg.)	0·680	0·818	1·005	s.
8	Mean efficiency index	0·226	0·156	0·226	n.s.
9	Relative growth rate as height ...	0·102	0·065	0·090	n.s.
	Relative growth rate as weight of:				
10	Stems	0·243	0·173	0·244	n.s.
11	Leaves	0·190	0·157	0·193	n.s.
12	Differential growth ratio between stem and leaf	1·32	1·06	1·30	—
13	Mean relative rate of leaf production	0·141	0·102	0·142	n.s.
14	Mean assimilation rate	0·394	0·321	0·383	—

*s. = significant; n.s. = not significant.

There seem to be three possible interpretations of these results:

(i) There may be practically no variety in genes controlling growth rates among different inbred races; this implies that the parent strains carry of the same factors for metabolic and developmental rates. If this were true crossing would not produce strains with relatively higher rates of development than existed in the parents, and there would be no segregation for these rates in generations subsequent to F_1.

(ii) Rates of development under experimental conditions may be determined by environment rather than by heredity, so that although two strains may differ in their hereditary potentiality as regards growth rates, these differences may be suppressed by the limiting influence of the environment. This would account for the fact that hybrids, despite heterosis, show no higher relative rates of development than their parents, and at the same time for the fact that hereditary differences in embryo size are apparent, for the primordia of embryos develop in a totally different environment. Suppression by the environment of potentialities for embryo growth is frequently encountered in the writer's work; the sizes of embryos from reciprocal crosses may differ greatly, and depend upon the size of the testa which encloses them.

On either of these interpretations the genes effective in determining size differences would operate early in the life cycle, and the manifestation of heterosis would depend on these genes. Data from two recent papers by Fabergé (1936*a*, *b*) may be interpreted in the same way. It was found that the relative growth rates of tetraploid tomatoes showed no evidence of segregation among different strains, and did not differ significantly from the relative growth rates of diploids. Neither Fabergé's results nor those of the present writer indicate which of these interpretations is the more probable.

(iii) The third possibility, which must not be excluded from consideration, is that there is some stimulus to development of the embryo consequent upon fertilization by a "foreign" male gamete. East originally advocated this view (East and Hayes 1912) and Jones (1917) is careful not to reject it in favour of his own hypothesis. One or two more recent works support implicitly the idea of a non-Mendelian cause of heterosis (Calkins 1919; Umeya 1930). The present writer's data are consistent with such a view, though they do not provide evidence in favour of it.

I am grateful to Professor Macgregor Skene for his criticism of the manuscript.

SUMMARY

The assumptions inherent in the polymeric-factor theory of size inheritance and the genetical theory of heterosis are discussed. Both theories make unsatisfactory postulates in order to account for the absence of dominance bias in F_2 populations, but in fact dominance bias can sometimes be demonstrated, and even when it is concealed the distribution

cannot be assumed to be symmetrical. Therefore the assumptions to explain this symmetry may be removed from the theories. When this is done the theories become consistent with one another and with the facts of size inheritance. They may, however, be stated in a form which emphasizes a fresh method of investigation; namely, the study of size inheritance through an analysis of the inheritance of the developmental processes which determine size.

Recent work on the physiology of heterosis, of which some new examples are given, is not at variance with a Mendelian hypothesis, but it is necessary to postulate either (i) that there is practically no variety in genes controlling growth rates among different inbred races, or (ii) that genetic differences between races, affecting rates of development, are suppressed by the limiting influence of the environment. There are no indications from the data as to which of these suggestions is the more probable.

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The Menstrual Cycle of the Primates

X—The Oestrone Threshold of the Uterus of the Rhesus Monkey

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Uterine bleeding occurs in spayed rhesus monkeys a few days after the cessation of a series of oestrin injections (Allen 1927). The fact that bleeding also follows either the removal of ovaries which do not contain functional corpora lutea or injury to large ovarian follicles (Allen 1927; van Wagenen and Aberle 1931) is now related to the occurrence of post-oestrin bleeding, and these various observations, together with parallel and in some cases long-established clinical findings, have been made the basis of what is known as the oestrin-withdrawal theory of menstruation. The experimental study of the menstrual cycle necessarily requires detailed knowledge of the varying reactions of the primate uterus to oestrin. The available observations on this question are very few, and for that reason the following study was undertaken.

1—Material and Methods

The experiments reported in this paper were performed on one prepubertal and on twenty-four adolescent and mature ovariectomized rhesus monkeys (*Macaca mulatta*). The maximum and minimum weights of the animals during the period of the experiments (March 1935–April 1937) are given in Table I.

Crystalline oestrone was used, dissolved either in nut oil, olive oil or water. Except for the two series of experiments in which a solution of oestrone in water was used and was given subcutaneously (p. 445), all injections were made intramuscularly. Observations of uterine bleeding were made by the vaginal lavage method, the vaginal contents being aspirated every day in each animal by means of a pipette filled with saline. In the tables the experiments are numbered by two sets of numerals separated by a point. The first figure is the animal's number, and the second the number of the particular series of injections it received.

TABLE I—UTERINE BLEEDING AFTER BILATERAL OVARIECTOMY

No. OM.	Max. body wt. g.	Min. body wt. g.	Ovaries removed, day of cycle	Uterine bleeding	Operative cycle days
75	5360	4600	1	Continued bleeding for 9 days	—
106	5660	4880	2	,, ,, ,, 11 ,,	—
180	4520	3930	2	,, ,, ,, 7 ,,	—
45	5120	3760	4	Bleeding 7 days after operation	10
97	5160	3700	4	No bleeding in 18 days after operation	—
53	4460	3560	8	,, ,, ,, 19 ,, ,, ,,	—
95	4740	3920	8	,, ,, ,, 18 ,, ,, ,,	—
70	7120	5140	9	Bleeding 11 days after operation	19
68	5790	5000	12	,, 5 ,, ,, ,,	16
3	6300	3530	16	,, 7 ,, ,, ,,	22
69	6240	5290	19	,, 2 ,, ,, ,,	20
55	3970	3560	22	,, 8 ,, ,, ,,	29
26	3620	2920	23	,, 5 ,, ,, ,,	27
64	4900	3350	24	,, 5 ,, ,, ,,	28
98	3800	2950	24	,, 2 ,, ,, ,,	25
71	5300	4480	33	,, 2 ,, ,, ,,	34
107	5280	3760	58	No bleeding in 14 days after operation	—
181	4120	3700	Unknown	Bleeding 3 days after operation	—
5	4620	3700	,,	,, 6 ,, ,, ,,	—
92	4980	3550	,,	,, 7 ,, ,, ,,	—
74	5550	4400	,,	,, 7 ,, ,, ,,	—
183	4640	4000	,,	,, 7 ,, ,, ,,	—
186	4170	3900	,,	No bleeding in 15 days after operation	—
78	3210	1790	Immature	—	—
96	3350	3200	,,	—	—

2—THE OCCURRENCE OF UTERINE BLEEDING AFTER BILATERAL OVARIECTOMY

Table I gives the day of the cycle on which the ovaries of the animals used in this study were removed. The occurrence of uterine bleeding after the operation and the number of days intervening between the two are also recorded. The data confirm and extend those published by Allen (1927) and by van Wagenen and Aberle (1931).

3—THE AMOUNT OF OESTRONE NECESSARY FOR UTERINE BLEEDING.

The concept of an oestrone threshold of the uterus in Primates is open to at least three interpretations. It might mean the least amount of the hormone necessary to produce, in the shortest possible time, definite signs of endometrial growth in a spayed monkey. It might mean the least amount of hormone, given over a stated number of days, that is needed to influence the endometrium sufficiently for the cessation of injections to be succeeded by uterine bleeding. Or it might mean the smallest dose which, given daily for an indefinite period, will maintain the endometrium without uterine bleeding occurring. The experiments reported in this section are concerned only with the second interpretation.

The course of events in the normal menstrual cycle, as reflected in the changes of the sexual skin in those primate species in which the main change is swelling, suggests that the degree of oestrogenic stimulation increases to a maximum at about the middle of the cycle and then suddenly decreases in intensity (Zuckerman 1930; Krohn and Zuckerman 1937). The modal cycle length in the rhesus monkey is 28 days (Hartman 1932; Zuckerman 1937*a*), and the mid-interval decline in oestrogenic stimulation, as indicated by sexual-skin changes, is related temporally to ovulation (Zuckerman 1930). Oestrin produces its positive morphological effects during the first half of the cycle, i.e. during approximately the first 14 days of the cycle. For that reason 14 days were chosen as the period during which to inject oestrone daily into spayed rhesus monkeys in order to discover the least amount of the hormone necessary to induce uterine bleeding. The method followed was to inject the animals with decreasing quantities of oestrone in successive 14-day courses of injection, intervals being left between courses to allow of "oestrin-withdrawal" bleeding. If bleeding occurred, a new course of injections was started on the second day of the phase of bleeding. If bleeding did not occur within the expected interval (see below) the animal was left for 26 days after its last injection before a new course of injections was begun. A similar plan was followed in all the experiments described in this paper, 40 days from the first injection of a course being regarded for the purposes of this study as the outer limit within which to allow for uterine bleeding.

The uterine-bleeding thresholds of fourteen animals as determined in this manner are given in Table II.

TABLE II—THE OESTRONE THRESHOLD FOR UTERINE BLEEDING IN DIFFERENT RHESUS MONKEYS

Amount daily for 14 days (I.U.)...	25	50	100	150
Total injected (I.U.)	350	700	1400	2100
Animals	OM. 64 OM. 97	OM. 71 OM. 3 OM. 68 OM. 92 OM. 181 OM. 186	OM. 5 OM. 69 OM. 70 OM. 75 OM. 95	OM. 45 OM. 74

Uterine bleeding occurred in no monkey injected daily with 12·5 I.U. of oestrone. Two animals not listed in Table II, OM. 106 and OM. 107, bled after fourteen daily injections of 150 I.U. The only prepubertal animal in the group that I studied bled after fourteen injections of 100 I.U., and thus had a lower uterine oestrin threshold than two of the mature animals. Allen and others (1936), who provide some observations on this question, believe that the threshold for bleeding is considerably higher in spayed immature than in spayed adult and adolescent rhesus monkeys (300 rat units as opposed to 81 rat units). The animals listed in Table I appear, however, to vary in threshold independently of weight and age. Unfortunately the rat units Allen and his co-workers used cannot be translated into the weight unit of the international standard except very approximately. Nevertheless, it would appear that some of my animals had lower thresholds than any investigated by them.

It should be noted that the uterine oestrone threshold of a monkey varies from time to time (see below).

4—THE LENGTH OF THE LATENT INTERVAL INTERVENING BETWEEN OESTRONE INJECTIONS AND UTERINE BLEEDING

(a) *Oil Solutions of Oestrone*

Table III gives the latent interval intervening between the onset of uterine bleeding and the cessation of a 14-day course of oestrone injections given intramuscularly in oil. In courses of injection in which the amount of oestrone given daily was 500 I.U. or more, the dosage was as a rule "stepped up" in a fixed way so that the animal received the maximum daily dose only during the last few days of the course. The total amounts of oestrone given in the standard courses of injection are stated in Table III.

The mean latent intervals for the groups in which there are eight or more observations vary somewhat, but statistical analysis fails to reveal any significant differences. No such differences appear, too, when the observations are grouped into the following three classes: 25–100 I.U.; 150–1000 I.U.; 2000–5000 I.U. The mean latent interval for all the seventy-four observations recorded in Table III is 8·1 days.

The amount of oil which acts as a vehicle for the oestrone appears (within the limits of the present experiments) to be without influence on the duration of the latent interval. Apart from several incidental observations to this effect, the following specific observations were made. OM. 97 was given 100γ oestrone daily for 14 days in four successive courses of injections, the hormone preparation used being of different dilution in each of the courses. The following results were obtained:

0·1 c.c. oil daily: uterine bleeding 7 days after end of injections
0·25 c.c. ,, ,, ,, ,, 9 ,, ,, ,, ,,
0·5 c.c. ,, ,, ,, ,, 13 ,, ,, ,, ,,
1·0 c.c. ,, ,, ,, ,, 10 ,, ,, ,, ,,

In view of the great variability in latency revealed in Table III, the differences between these figures cannot be regarded as more than "chance" variations.

Dilutions of oestrone of the order indicated in the second, third and fourth experiments referred to above were never used in practice for the purpose of administering as much as 100γ of oestrone. In general the solutions employed were as concentrated as was convenient for the purposes required, and the same dilutions were used for comparable daily injections of oestrone throughout the series of experiments described in this paper.

(*b*) *Aqueous Solutions of Oestrone*

The possibility that the latent interval preceding uterine bleeding is longer when oil rather than aqueous solutions of oestrone are used was investigated. Table IV gives the records of sixteen experiments in which different amounts of oestrone dissolved in water at a concentration of 1000 I.U./c.c. were given subcutaneously for 14 days. If anything, the latent intervals in this group of experiments are somewhat longer than in those listed in Table III. The mean of the sixteen intervals recorded is 9 days. The latency appears to increase as larger amounts of oestrone and larger amounts of the solution are injected. As the problem raised by this

TABLE III—THE LATENT INTERVAL BEFORE UTERINE BLEEDING AFTER FOURTEEN DAILY INTRAMUSCULAR INJECTIONS OF OESTRONE IN OIL. THE EXPERIMENT NUMBER IS GIVEN AS THE NUMBER OF THE ANIMAL FOLLOWED BY THE NUMBER OF THE PARTICULAR SERIES OF INJECTIONS ADMINISTERED TO IT

Max. daily dose (I.U.) ...	25		50		75		100		150		200		400		500		850		1000		2000		3000		4000		5000	
Total in 14 days (mg.) ...	0·035		0·070		0·105		0·140		0·210		0·280		0·560		0·580		0·950		1·040		1·800		2·700		74...3·900 181...5·600		4·600	
No. of exp. and latent interval in days ...	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days	Exp. no.	Days
	64.9	9	3.22	11	97.4	7	3.20	8	3.19	7	97.1	7	5.15	8	45.1	12	45.14	7	3·18	8	3.17	7	5.16	11	74.16	9	5.9	15
	97.6	12	64.7	7			70.9	9	45.7	7					68.8	7	92.12	6	5.14	9	75.14	7	45.16	8	181.2	8	75.20	8
			68.5	3			75.8	5	45.17	8					69.6	6			68.9	7			55.1	6	3.25	6		
			71.1	9			78.14	6	70.1	9					70·8	8			69·8	7			64.8	10				
			92.16	10			92.15	6	71.6	8					74.11	8			71.7	9			68.7	8				
			97.5	11			95.9	8	74.12	9					75.16	6			74.13	9			70.3	12				
			181.4	6			97.3	7	92.14	8					92.13	6			78.3	9			71.5	11				
			186.4	6			181.3	4	97.2	6					97.18	7			78.4	9			74.9	9				
																			97.7	7			75.4	7				
																			97.8	9			75.5	7				
																			97.9	13			92.11	9				
																			97.10	10			95.10	14				
																			183.1	7			97.15	8				
																							186.3	8				
																							3.24	8				
Mean		10·5		7·9		7·0		6·6		7·8		7·0		8·0		7·5		6·5		8·7		7·0		9		8		11·5

observation lay outside the scope of the present investigation, it was not pursued further.

It may be concluded that in so far as the duration of the latent interval before uterine bleeding constitutes an index of the utilization of oestrone, oil solutions of the hormone given intramuscularly do not retard its absorption and effective functioning.

TABLE IV—THE LATENT INTERVAL BEFORE UTERINE BLEEDING AFTER FOURTEEN DAILY SUBCUTANEOUS INJECTIONS OF AN AQUEOUS SOLUTION OF OESTRONE

Max. daily dose (I.U.) ...	100		150		1000		3000	
Total in 14 days (mg.) ...	0·14		0·21		1·04		2·7	
No. of exp. and latent interval in days	Exp.	Days	Exp.	Days	Exp.	Days	Exp.	Days
	5.10	8	64.4	7	45.9	7	3.13	11
	68.1	8	69.2	6	64.3	9	70.4	15
	69.1	6	106.2	7	75.9	7	75.6	18
			107.2	5	92.7	8	106.1	9
							107.1	14
Mean		6·25		7·3		7·75		13·4

5—VARIATIONS IN THE INTERVAL PRECEDING UTERINE BLEEDING IN RELATION TO THE DURATION OF INJECTIONS

Apart from the experiments listed in Tables III and IV, several were performed in which oestrone was given for longer periods than 14 days. In almost all of these experiments injections were continued for 40 days, the amount of oestrone given daily being much reduced after the 14th day. The data relating to these long periods of injection are given in Table V.

The most frequently occurring latent interval in the group of 40 days injections is 6 days. Owing to the fact that a steady level of oestrone was not maintained in most of these experiments, this finding can only be regarded as suggestive of the conclusion that a shorter interval precedes uterine bleeding after longer than after shorter courses of injection. Allen and others (1936) believe that longer latent periods occur before uterine bleeding when oestrone is administered for periods shorter than 10 days. The first observation listed in Table V does not lend support to this view.

6—THE DURATION OF OESTRIN-WITHDRAWAL UTERINE BLEEDING

The mean duration of uterine bleeding for the different groups of experiments listed in Table III differ somewhat, but statistical analysis reveals no

Table V—Latent Interval preceding Bleeding and Duration of Bleeding after Courses of Injections of less than and more than 14 Days

Exp. no.	Max. dose daily in 1st 14 days I.U.	Daily dose day 15 onwards I.U.	Total no. of days	Total amount oestrone mg.	Latency before bleeding days	Duration of bleeding days
55.2	500	—	5	0·25	8	—
78.2	1000	1000	18	1·80	10	—
78.1	1000	1000	19	1·90	6	—
3.8	3000	500	40	3·85	5	4
3.9	3000	500	40	3·85	5	4
3.11	2000	500	40	3·04	5	5
3.16	1000	150	40	1·43	6	3
5.6	3000	500	40	3·80	7	3
5.7	3000	500	40	3·80	7	4
5.8	1000	500	40	2·10	6	3
5.11	3000	100	40	2·96	5	5
45.8	1000	200	40	1·52	4	4
64.1	3000	500	40	3·80	11	3
64.2	1000	500	40	2·20	6	3
64.10	3000	300	40	3·48	6	4
69.5	500	150	40	1·09	5	4
70.5	3000	200	40	3·22	6	3
71.4	1000	150	40	1·43	6	4
74.1	4000	300	40	4·07	7	4
74.2	1000	100	40	1·27	6	3
74.3	3000	500	40	3·75	7	4
74.5	3000	200	40	3·22	7	2
74.6	500	500	40	1·75	8	3
74.10	500	150	40	1·09	7	4
75.1	5000	300	40	4·87	6	4
75.7	2000	500	40	2·99	6	3
75.13	1000	100	40	1·30	6	2
92.5	850	300	40	1·73	6	4
92.6	1000	500	40	2·15	6	4
95.1	5000	200	40	4·60	6	3
95.2	3000	150	40	3·09	5	4
95.3	3000	500	40	3·85	10	4
95.4	500	500	40	1·80	7	5
95.5	1000	500	40	2·05	7	3
95.6	3000	200	40	3·22	6	3
95.7	1000	150	40	1·49	6	6
97.11	3000	150	40	3·09	6	4
97.12	1000	100	40	1·36	6	3
180.1	1000	1 mg.	64	25·30	10	8

significant variations. Bleeding does not appear to be prolonged after long periods of injection (Table V). On the basis of many fewer observations, Allen and others (1936) state, however, that phases of uterine bleeding following oestrone injections are shorter, and the amount of flow is decreased, as the dosage and the period of injections is decreased. It is possible that this view applies only to periods of injection shorter than 10 days.

TABLE VI—DURATION OF BLEEDING (IN DAYS) IN EXPERIMENTS LISTED IN TABLE III. THE FIGURES REFER TO THE EXPERIMENTS IN THE ORDER THEY ARE GIVEN IN THAT TABLE

	25 I.U.	50 I.U.	75 I.U.	100 I.U.	150 I.U.	200 I.U.	400 I.U.	500 I.U.	850 I.U.	1000 I.U.	2000 I.U.	3000 I.U.	4000 I.U.	5000 I.U.
	4	2	6	3	3	5	4	6	4	4	5	7	3	5
	2	3		3	4			4	6	4	2	5	9	—
		2		2	5			5		6		3	—	
		2		3	4			5		5		6		
		4		6	4			3		6		6		
		3		3	2			4		5		2		
		5		6	4			5		—		8		
		6		6	4			6		—		3		
										4		3		
										4		3		
										5		3		
										4		—		
										7		5		
												9		
												—		
Mean	3·0	3·4	6·0	4·0	3·8	5·0	4·0	4·8	5·0	5·0	3·5	4·8	6·0	5·0

It should be noted that a new course of injections was generally begun on the second day of each phase of bleeding. In comparison with the observations published by Allen and others (1936), the duration of bleeding in none of the experiments recorded above appears, however, to have been curtailed. It should be noted too that the duration of flow in the menstrual cycle of normal rhesus monkeys is usually from 4 to 5 days (Corner 1923; Hartman 1932) and that oestrogenic stimulation, as indicated by sexual-skin changes, begins at the start of the normal cycle.

7—THE VARIABILITY OF INDIVIDUAL ANIMALS

(*a*) *Individual Variations in the Latent Interval preceding Uterine Bleeding*

Two animals were twice given the same 14-day course of injections. OM. 45 was given 150 I.U. of oestrone daily on each occasion. The latent interval was 7 days after the first and 8 after the second course. OM. 75 was given up to 3000 I.U. in each of the courses. The latent interval before bleeding was 7 days on both occasions. The same animal manifested a latent interval of 18 days when injected with a similar amount of oestrone dissolved in water (Table IV).

The mean latent period before bleeding in different monkeys is given in Table VII. There does not appear to be any significant difference in respect of this interval between the several animals listed.

TABLE VII—MEAN LATENT INTERVAL PRECEDING UTERINE BLEEDING IN DIFFERENT ANIMALS

Animal OM.	Range days	Mean days
3	5–11	7·3
5	5–15	8·6
45	4–12	7·5
64	6–11	8·1
68	3–8	6·6
69	5–7	6·0
70	6–15	9·8
71	8–11	9·25
74	6–9	7·8
75	5–18	7·5
92	6–10	7·2
95	5–14	7·7
97	6–13	8·3

(b) *Individual Variations in the Duration of Bleeding*

The data given in Table VIII suggest that individual differences exist between animals in regard to the length of time uterine bleeding lasts, some monkeys bleeding for much shorter periods than others. Similar variability occurs in the menstrual cycles of normal animals (Hartman 1932).

8—THE LATENT PERIOD PRECEDING UTERINE BLEEDING

Eighteen experiments were performed in order to discover the point during the post-injection latent period at which the processes leading to endometrial breakdown cease to be reversible. Monkeys were injected for 14 days with oestrone, the maximum amounts given varying between 500 and 3000 I.U. daily. No injections were given for from 3 to 6 days, beginning on the 15th day. Injections were then restarted at a constant level of 500 I.U. daily, an amount sufficient to maintain an interval endometrium without the occurrence of uterine bleeding. Where no bleeding

TABLE VIII—DURATION OF UTERINE BLEEDING IN DIFFERENT ANIMALS

Animal OM.	Range days	Mean days	Mode days
3	2–10	4·3	4
5	2–7	4·1	4
45	2–8	4·9	5
64	3–6	3·9	3 : 4
68	2–7	4·5	4
69	3–5	3·8	3
70	2–5	3·4	3
71	2–8	5·3	—
74	2–5	3·2	3
75	2–4	2·9	3
78	3–7	4·8	5
92	3–6	4·4	4
95	3–6	4·0	3
97	2–6	4·2	4
106	3–4	3·5	—
107	3–4	3·7	—

occurred, injections were continued to the 40th day of the experiment. The results of these experiments are given in Table IX.* The table also gives the latent intervals preceding bleeding as observed in the same animals in control experiments after injections of the same amounts of oestrone for the first fourteen days. In the cases where the actual "control" latent interval is unknown, mean figures from Tables III and IV are employed, the instances in question being marked with an asterisk to show that this has been done.

The data listed in Table IX show clearly that even when injections of oestrone are restarted after the middle of the latent period following a previous course of injections, the uterine bleeding that would normally occur can be averted. The data suggest that it is only in the last phase of the interval preceding bleeding that the processes leading to endometrial breakdown are irreversible. The turning-point is probably the time when the concentration of oestrone in the body falls to a subthreshold level.

Allen (1928) has stated that an extravasation of red cells into the endometrial stroma occurs as early as one day after the end of oestrin injections. No such extravasations are present in three of my specimens which were autopsied the day after the end of a course of such injections (OM. 96 after 14 days of injection, OM. 53 after 40 days and OM. 98 after 365 days). In the normal cycle, sudden extravasations of red cells occur only just before menstruation sets in (Bartelmez 1937, quoting Markee).

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* A preliminary statement has been made about all but two of these experiments (Zuckerman 1936*a*).

TABLE IX—THE LATENT INTERVAL PRECEDING UTERINE BLEEDING

Exp.	First 14 days max. daily dose I.U.	Oestrone solvent	No. of days uninjected after 14th day	Normal latent period	Result
3.8	3000	Oil	3	8	No bleeding
3.9	3000	Water	3	11	,, ,,
95.3	3000	,,	3	13*	,, ,,
5.6	3000	Oil	4	11	,, ,,
5.7	3000	Water	4	13*	,, ,,
64.1	3000	,,	4	13	,, ,,
74.3	3000	Oil	5	9	,, ,,
55.1	3000	,,	5	6	Bleeding, day 20
75.5	3000	,,	6	7	,, ,, 21
3.11	2000	,,	4	7	No bleeding
75.7	2000	,,	5	7	,, ,,
5.8	1000	,,	4	9	,, ,,
64.2	1000	,,	4	9*	,, ,,
3.10	1000	,,	5	8	Bleeding, day 20
92.6	1000	,,	5	8	No bleeding
95.5	1000	,,	5	8·7*	,, ,,
95.4	500	,,	4	7·5*	,, ,,
74.6	500	,,	5	8	,, ,,

Apart from revealing the fact that the processes leading to oestrin-withdrawal uterine bleeding can be checked, the experiments recorded above leave the question of the actual nature of these processes unsolved.

9—THE AMOUNT OF OESTRONE NECESSARY TO MAINTAIN AN OESTRONE-STIMULATED ENDOMETRIUM

As noted earlier on, one interpretation of the concept of an oestrone threshold of the primate uterus is the smallest dose which, given daily for indefinite periods, will maintain the endometrium in a phase of growth without uterine bleeding occurring. This interpretation was tested in an experiment in which a spayed monkey, OM. 98, was injected daily for 365 days with 1000 I.U. of oestrone in oil. Uterine bleeding did not occur at any time during the course of the experiment. At its close the endometrium was in a condition of pronounced cystic hyperplasia (Zuckerman 1937*b*).

Similarly, bleeding did not occur during several experiments on different animals which during the course of 40 days were never injected with less than 500 I.U. of oestrone daily (cf. Table V). It occurred only once (45·15) during the course of injections in another series of five experiments in which the daily dose of oestrone was dropped from a high level on the 14th day to 300 I.U. daily on the 15th and then maintained at that lower level till the 40th day. Uterine bleeding is to be expected during such courses of injections only when the daily injection is dropped on the 15th day to the region of 150 I.U. and less (cf. Table V and following paper).

The level at which oestrone is unable to maintain the endometrium in a phase of growth without uterine bleeding occurring appears to be about that of the threshold dose which given daily over a period of 14 days will influence the endometrium sufficiently for the cessation of injections to be followed by uterine bleeding. Thus OM. 78, an immature spayed monkey, bled 6 days after a 14-day course of 100 I.U. oestrone daily. The animal was also injected daily for 365 successive days with the same amount of oestrone. In spite of the continuous injections, eight separate phases of bleeding occurred during the experiment. The animal was clearly not being given enough hormone to maintain its uterus indefinitely in a phase of growth. Uterine bleeding occurs during the continuous administration of oestrone only when the level of oestrone falls below a daily threshold value, and this value is above the minimum amount which given daily over a period of 14 days will produce enough growth, as noted above, for the cessation of injections to be succeeded by uterine bleeding.

The intervals at which uterine bleeding occurred during the year OM. 78 was injected daily are of considerable interest. The first interval is counted as from the first day of injections to the first day of the first phase of bleeding, the second interval as from the second day of the first phase of bleeding to the first day of the second phase, and so on.

Phases of bleeding	Intervals between bleeding days	Duration of bleeding days
1	48	5
2	43	3
3	35	3
4	46	6
5	46	7
6	31	6
7	40	4
8	41	5
	35 incomplete	

The mean length of the eight intervals is 41·25 days. It is almost inconceivable that bleeding could have recurred eight times every 5–7 weeks purely as a matter of chance. The fact that it did arise raises an issue of great importance in the consideration of cyclical phenomena.

DISCUSSION

In view of the fact that uterine bleeding will not occur in spayed monkeys when under continuous injection with an amount of hormone that is well above threshold value (e.g. OM. 98), the successive phases of bleeding in OM. 78 must be interpreted as reflections of successive falls in the degree of oestrogenic stimulation experienced by the animal. The possibility that these falls may have been due to the influence of some accessory ovarian tissue was excluded. Close search at autopsy failed to reveal any trace of such tissue anywhere in the pelvic or abdominal cavities. When the animal was spayed the infundibulo-pelvic ligaments, the distal parts of the ovarian vessels, and the lateral halves of both Fallopian tubes were removed. The stumps of the Fallopian tubes, the broad ligaments and the distal ends of the ovarian vessels were serially sectioned and examined microscopically in case some minute fragments of accessory ovarian tissue had been overlooked. None was found.

Since the monkey was being continuously injected with the same amount of hormone daily (100 I.U.), (an amount sufficient to maintain the endometrium in the intervals between the phases of bleeding), it follows that rhythmical changes occurred within the animal itself of a kind that decreased the effectivity of the hormone, i.e. of a kind that rhythmically raised the animal's oestrone threshold. Such an effect could come about either as a result of "inherent" rhythmical variations in the responsiveness of the endometrium to oestrone or as a result of the rhythmical liberation into the blood stream of some substance which is able to neutralize the effects of oestrin (cf. the antagonism of oestrin and progestin (Leonard, Hisaw and Fevold 1932; Allen and Meyer 1935) and that of oestrone and male hormone (Zuckerman and Parkes 1936; Zuckerman 1936 *b*). The possibility that the pituitary and adrenals are in some way concerned is being investigated.

It is of considerable interest that the oestrone threshold was rhythmically raised in OM. 78, since the animal was of prepubertal age throughout the course of the experiment, judging by its weight and by its dentition. This fact suggests that the accessory reproductive organs are subjected to some kind of rhythmical control long before the ovaries undergo rhythmical morphological changes.

A further conclusion that follows from this observation is the possibility that variations in the latent interval which intervenes between the cessation of injections of oestrone and the onset of uterine bleeding may in some way be determined by individual fluctuations in the responsiveness of the endometrium to oestrone. It is highly unlikely that courses of oestrone injections would always end in different animals (or in the same animal at different times) at the same point in relation to phases in which the uterine threshold to oestrone was raised, and the effects of the oestrone that had been injected would be thus correspondingly prolonged or retarded. It also appears that the variations which are observed in the same animal's oestrin threshold from time to time are determined by the different times injections are given in relation to "inherent" rhythmical fluctuations in the animal's sensitivity to the hormone. This question is discussed further in the following paper.

SUMMARY

Spayed rhesus monkeys vary with respect to the least amount of oestrone which when administered over a period of 14 days is required to influence the endometrium sufficiently for the cessation of injections to be followed by uterine bleeding. The lowest daily dose ranges from 25 to 150 I.U. The threshold in adolescent and mature animals at any given time appears to vary independently of body weight and age.

Both the latent interval preceding bleeding and the duration of bleeding vary from time to time and from animal to animal. They do not appear to vary significantly in relation to the amount of hormone that is administered over a period of 14 days. Like normal animals, some spayed monkeys bleed longer than others.

If injections of oestrone are restarted at any point before or just after the middle of a latent interval which follows a course of previous injections, the bleeding that would otherwise occur can be averted. This observation is taken to mean either that the effects of a series of injections are prolonged some days after the cessation of injections or that the processes leading to endometrial breakdown are reversible in their earlier stages.

Uterine bleeding did not occur during a year's course of continuous daily injections of an amount of oestrone that was well above the threshold value.

Bleeding occurred eight times at intervals of from 5 to 7 weeks in an animal that was injected daily for a year with a constant amount of oestrone which was about the threshold value. This observation is taken to mean that in spayed animals (presumably in normal ones too) the oestrin threshold rises and falls in a rhythmical manner for reasons unassociated with cyclical changes in the gonads. The significance of this observation,

which introduces a new element into the consideration of the menstrual cycle, is discussed in relation to other phenomena.

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The Menstrual Cycle of the Primates

XI—The part played by Oestrogenic Hormone in the Menstrual Cycle

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[Plates 17, 18]

The following paper is a record of a series of experiments which were planned for the purpose of discovering conditions of oestrogenic stimulation under which uterine bleeding could be made to occur in ovariectomized rhesus monkeys at approximately 4-weekly intervals. The theoretical basis of these experiments was an hypothesis of the menstrual cycle that had been derived deductively from observational data (Zuckerman 1930).

PRELIMINARY OBSERVATIONS

Ovulation does not occur in every menstrual cycle either in man (Bartelmez 1937), in common macaques (van Herwerden 1905), in rhesus monkeys (Corner 1923; Hartman 1932) or, as I have recently found, in baboons. The menstrual rhythm, however, is not influenced by the occurrence or non-occurrence of ovulation. In cycles in which ovulation does not occur, the sex-hormone influencing the endometrium can only be, so far as is at present known, the oestrogenic hormone elaborated by the ovaries, for a corpus luteum does not develop in such cycles. Moreover the endometrium is neither directly influenced by gonadotropic hormones nor is the pituitary necessary for ovarian hormones to exercise their endometrial effects (Saiki 1932; Hisaw 1935; Smith, Tyndale and Engle 1936). The menstrual rhythm is presumably maintained by cyclical fluctuations in the degree of oestrogenic stimulation, and such fluctuation must at present be regarded as the basic mechanism of the menstrual cycle.

Examination of the ovaries of a group of Hamadryas baboons (*Papio hamadryas*), animals in which the sexual-skin cycle is very conspicuous and definite, has recently shown that the sexual-skin changes are the same in anovular cycles as in cycles in which ovulation occurs. The less definite rhythm of changes in the sexual skin of rhesus monkeys is also uninfluenced by the occurrence or non-occurrence of ovulation (Hartman 1932). These observations imply that in monkeys (as in mice, Parkes 1926) the cycle of oestrogenic stimulation is not obviously affected by the more conspicuous morphological changes in the ovaries. The degree of stimulation in normal monkeys increases gradually to a maximum during the first half of the cycle and then suddenly declines.

EXPERIMENTAL

Details about the spayed rhesus monkeys (*Macaca mulatta*) used in this study* are given in the preceding paper. Oil solutions of crystalline oestrone were used, and all injections were given intramuscularly. Observations on uterine bleeding were made by the vaginal lavage method.

Spayed monkeys were injected daily with increasing amounts of oestrone for 14 days, the maximum dose varying in different experiments. During this period, which corresponds to the earlier half (preovulation follicular phase) of the normal cycle, an interval endometrium was built up under the influence of the oestrone (fig. 1, Plate 17). On the 15th day of each experiment the amount of oestrone given daily was reduced to and maintained at a fixed low level, which varied from experiment to experiment. If uterine bleeding occurred during the course of the low level of injections, the experiment was ended and a new one begun on the second day of bleeding. The interval between and including the first day of injections and the first day of bleeding demarcated an "artificial cycle". If bleeding did not occur, injections were stopped on the 41st day, and the animals were not injected again until after the occurrence of oestrin-withdrawal bleeding. Experiments in which "artificial cycles" were not demarcated are indicated in the following tables by the figures 40 + 4, 5, 6, or 7, depending on the number of days intervening between the cessation of injections and the onset of uterine bleeding. All other figures in the columns headed "artificial cycle length" represent instances in which bleeding occurred during the course of injection of the particular low dose given in the latter half of the experiment concerned. The tables also give the oestrin thresholds for each animal, as determined previously (see preceding paper), as well as the length of "artificial cycle" that would have occurred had the animals in each experiment not been given any oestrone after the 14th day. Each of these "control artificial cycles" thus comprises the initial 14 days of injection plus the latent interval that intervened between the cessation of injections and the onset of bleeding in control experiments (see Tables III and IV, preceding paper; also fig. 2 this paper). Where actual control figures for a particular animal are unknown, the mean figures taken from the tables in the preceding paper have been used. Such instances are marked in the tables with an asterisk. The columns headed "% fall" show the extent of the reduction of the dose of oestrone on the 15th day.

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* A preliminary statement was made about a few of the earlier of these experiments (Zuckerman 1936*b*). Some of the views stated then have had to be modified.

The total amount of oestrone given during the first 14 days of each experiment varied as follows according to the maximum daily dose given:

Maximum daily dose I.U.	Total injected mg.	Maximum daily dose I.U.	Total injected mg.
400	0·56	2000	1·80
500	0·58	3000	2·70
850	0·95	4000	3·30
1000	1·04	5000	4·10

Tables I, II, III and IV give the results of sixty-two experiments of the kind described above.

TABLE I—"ARTIFICIAL MENSTRUAL CYCLES" IN SPAYED RHESUS MONKEYS

Exp. no. (OM.)	Max. dose 1st 14 days I.U.	Dose day 15 onwards I.U.	% fall	Artificial cycle length days	Control artificial cycle days	Daily threshold dose I.U.
92.5	850	300	35·3	40 + 6	20	50
64.10	3000	300	10	40 + 6	24	25
74.1	4000	300	7·5	40 + 7	23	150
75.1	5000	300	6·0	40 + 6	22	100
45.15	3000	300	10	32	22	150
92.4	850	250	29·5	26	20	50
45.8	1000	200	20	40 + 4	21	150
3.5	2000	200	10	38 incomplete	21	50
95.6	3000	200	6·7	40 + 6	28	100
74.5	3000	200	6·7	40 + 7	23	150
70.5	3000	200	6·7	40 + 6	26	100
3.6	4000	200	5·0	37 incomplete	20	50
95.1	5000	200	4·0	40 + 6	29*	100
92.3	850	200	23·5	25	20	50
92.2	850	175	20·5	25	20	50
75.3	2000	175	8·8	26	21	100

Table II—"Artificial Menstrual Cycles" in Spayed Rhesus Monkeys

Exp. no. (OM.)	Max. dose 1st 14 days I.U.	Dose day 15 onwards I.U.	% fall	Artificial cycle length days	Control artificial cycle days	Daily threshold dose I.U.
95.2	3000	150	5	40 + 5	28	100
53.1	3000	150	5	40 incomplete	23*	—
97.11	3000	150	5	40 + 6	22	25
3.2	3000	150	5	28	22	50
3.4	2000	150	7·5	29	21	50
71.4	1000	150	15	40 + 6	23	50
95.7	1000	150	15	40 + 6	23*	100
3.16	1000	150	15	40 + 6	22	50
68.4	1000	150	15	25	21	50
45.4	850	150	17·7	28	21	150
92.1	850	150	17·7	28	20	50
69.5	500	150	30	40 + 5	20	100
74.10	500	150	30	40 + 7	22	150
68.6	500	150	30	36	21	50
45.5	500	150	30	29	26	150

Table III—"Artificial Menstrual Cycles" in Spayed Rhesus Monkeys

Exp. no. (OM.)	Max. dose 1st 14 days I.U.	Dose day 15 onwards I.U.	% fall	Artificial cycle length days	Control artificial cycle days	Daily threshold dose I.U.
5.11	3000	100	3·3	40 + 5	25	100
70.2	3000	100	3·3	32	26	100
75.10	3000	100	3·3	30	21	100
92.8	3000	100	3·3	29	23	50
68.2	3000	100	3·3	25	22	50
3.14	3000	100	3·3	21	22	50
75.2	2000	100	5	23	21	100
74.2	1000	100	10	40 + 6	23	150
97.12	1000	100	10	40 + 6	24	25
75.13	1000	100	10	40 + 6	21	100
45.11	1000	100	10	26	21	150
45.13	500	100	20	27	26	150
92.10	500	100	20	26	20	50
68.3	500	100	20	24	21	50
75.15	500	100	20	23	20	100

TABLE IV—"ARTIFICIAL MENSTRUAL CYCLES" IN SPAYED RHESUS MONKEYS

Exp. no. (OM.)	Max. dose 1st 14 days I.U.	Dose day 15 onwards I.U.	% fall	Artificial cycle length days	Control artificial cycle days	Daily threshold dose I.U.
74.7	3000	50	1·7	31	23	150
64.5	3000	50	1·7	29	24	25
71.2	3000	50	1·7	28	25	50
106.3	3000	50	1·7	26	23	—
3.12	3000	50	1·7	21	22	50
97.13	1000	50	5	35	24	25
64.6	1000	50	5	32	23	25
69.4	1000	50	5	26	21	100
5.3	1000	50	5	25	23	100
106.4	1000	50	5	23	23*	—
70.6	500	50	10	29	22	100
45.12	500	50	10	23	26	150
74.8	500	50	10	23	22	150
45.2	500	50	10	23	26	150
75.12	500	50	10	20	20	100
5.4	400	50	12·5	27	22	100

RESULTS

Uterine bleeding occurred during the period of injections in thirty-nine of the sixty-two experiments. In the remaining twenty-three the level of oestrone had presumably not been lowered sufficiently at the particular time in which the experiment was performed (see below) for bleeding to occur. Only one of five animals (45.15, Table I) bled during the course of injections of 300 I.U. Bleeding occurred during only one of eight experiments in which the low level of oestrone was 200 I.U., and in seven of fifteen experiments in which the low level was 150 I.U. Bleeding occurred in most experiments in which the low level was 100 I.U. and in all in which it was 50 I.U. About 200 I.U. of oestrone a day is sufficiently above the threshold range in most animals for uterine bleeding not to occur during a period of 40 days of injections.

The cyclical occurrence of fluctuations in the responsiveness of an animal to oestrone has been demonstrated in the preceding paper. Such fluctuations are also revealed in the present series of experiments. Thus in experiment 45.15 (Table I) an animal bled during the course of daily injections of 300 I.U. In 45.8 (Table I) it failed to bleed during the course of injections of 200 I.U. Presumably the inability of the higher dose to maintain the endometrium in a phase of growth was due to the fact that the animal's oestrone threshold rose during the course of that series of injections. Similarly OM. 75 failed to bleed during the course of daily injections of 100 I.U. (75.13, Table III) but bled when injected with 175 I.U. (75.3, Table I).

Analysis of the relation between the oestrone thresholds of the different animals, as and when determined previously, and the levels of oestrone on which they either bled or did not bleed in the present experiments reveals some striking facts regarding fluctuations in the sensitivity of the different animals to the hormone.

Table V—Uterine Bleeding during the course of Injections in Relation to the Tested Oestrone Threshold of the Uterus. The Animals are arranged in the order of Increasing Amounts of the Threshold Dose on which Bleeding occurred

Animal OM.	Tested threshold I.U. daily × 14	Bleeding (tested threshold I.U. ×)	No bleeding (tested threshold I.U. ×)
95	100	—	1½ : 1½ : 2 : 2
74	150	⅓ : ⅓	⅔ : 1 : 2 : 1⅓
69.	100	½	1½
5	100	½ : ½	1
70	100	½ : 1	2
71	50	1	3
75	100	½ : 1 : 1 : 1 : 1¾	1 : 3
45	150	⅓ : ⅓ : ⅔ : ⅔ : 1 : 1 ; 2	1⅓
97	25	2	4 : 6
64	25	2 : 2	12
3	50	1 ; 2 : 3 : 3	3 : 4 : 4
68	50	2 : 2 : 3 : 3	—
92	50	2 : 2 : 3 : 4 : 5 : 3½	6

The table divides the data for each animal into two groups depending on whether or not the animal bled during the course of injections. The figures give the relation of the particular low level of oestrone on which an animal bled to the threshold value as determined previously (called "tested threshold" hereafter).

Even though the determination of the oestrin threshold of the uterus was not carried to any final point,* it is clear that in some instances amounts of oestrone in considerable excess of the tested threshold dose will fail to prevent uterine bleeding (e.g. OM. 92). The data towards the head of the table show, however, that for the most part the threshold values are valid, since in only one instance out of twelve did an animal fail to bleed during the course of injections of subthreshold doses. The same data show that bleeding may or may not occur when oestrone is administered at exactly the tested threshold level, and that bleeding is generally inhibited by slightly higher levels of oestrone. In the circumstances it appears that the instances (grouped at the foot of the table) where animals have bled during the course of injection of doses of oestrone which were three, four and five times the tested threshold value must be explained on the grounds that the sensitivity of the animals at the particular time their oestrone thresholds were determined were considerably different from and much lower than they were at the particular times at which the present experiments were carried out. It is particularly striking that the tested thresholds in these same animals at the foot of the Table are with one exception the lowest in the series. In view of the strong presumption that the threshold fluctuates in a cyclical manner (OM. 78, preceding paper), it follows that by chance some of the animals were at the bottom and others at the top of a curve of sensitivity to oestrone when their thresholds were determined.

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* The animals were tested with the following doses of oestrone given daily over a period of 14 days: 150, 100, 50, 25 and 12·5 I.U.

The fact that some of the animals bled during courses of injections in which three to five times the threshold value of oestrone was given daily suggests too that the "inherent" sensitivity of animals to oestrone fluctuates over a fairly wide range (from about 50 to 250 I.U.). "Oestrin-withdrawal" bleeding will *not* occur during the continuous injection of a constant level of oestrone only when the amount of hormone given daily is above the limits within which the threshold fluctuates.

Uterine bleeding following the administration of high doses of oestrone in the first 14 days of a course of injections is definitely delayed if from the 15th day onwards the daily dose of oestrone is reduced to some level within the range of fluctuation of the oestrone threshold. Thus the thirty-nine "artificial cycles" which occurred in the sixty-two experiments recorded in Tables I–IV ranged from 20 to 35 days, the mean being 26·77 days. The thirty-nine "control artificial cycles" (period demarcated by the first day of an identical 14-day course of injections and the first day of the subsequent phase of uterine bleeding) range from 20 to 26 days, the mean being 22·31 days. The difference between the two means is 4·46 ± 0·66 and is obviously significant.

Four instances occur in which the length of the artificial cycles is greater than their control cycles (3.14, Table III; 3.12, 45.12 and 45.2, Table IV). These anomalies were probably due to fluctuations occurring in the oestrone thresholds during the periods of injection, the "artificial cycles" presumably terminating at a time when the thresholds rose. The fact that amounts of oestrone which were below the tested threshold level were able in certain of the present experiments to retard the occurrence of uterine bleeding can be explained in a corresponding way.

The relative extent of the 15th-day fall in the amount of oestrone administered appears to have no determining effect on the occurrence of bleeding. Bleeding apparently takes place no more readily when the fall is relatively great (e.g. to 5 % of the previous level) than when it is relatively small (e.g. to 30 %). On the other hand, the amount of oestrone given in the first 14 days of each experiment does appear to influence bleeding slightly. Bleeding occurred sooner when the total given in the first 14 days was relatively small. Thus the mean of twelve artificial cycles in which 3000 I.U. was the maximum daily dose of oestrone in the first 14 days is 27·7 days, while 25·8 days is the mean of twelve cycles in which 500 I.U. was the maximum dose.

The tendency for uterine bleeding to occur becomes greater as the daily dose of oestrone is dropped on the 15th day to the lower levels of the threshold range. Thus bleeding occurred in sixteen of sixteen experiments when the dose was dropped to 50 I.U., in eleven of fifteen when it was dropped to 100 I.U. and only in seven of fifteen when it was dropped to 150 I.U. Moreover, the artificial cycles which are demarcated on the higher threshold doses appear to be longer than those which occur on the lower doses. The mean length of artificial cycle in the series in which 50 I.U. was the low level of oestrone is 26 days. It is 26 days in the series in which the low level was 100 I.U., and 28 days in the twelve instances in which the level was 150 I.U. or more. The tendency for artificial cycles to be shorter on the lower levels of oestrone is demonstrated by the last group of experiments listed in Table IV.

Discussion

As pointed out above, the curve of oestrogenic stimulation follows the same course in anovular menstrual cycles as in cycles in which ovulation occurs. On the basis of the experiments reported in the preceding paper, it would therefore be expected that uterine bleeding would occur about the 22nd or 23rd day of anovular cycles if the degree of oestrogenic stimulation were to fall to zero about the 15th day (which is the approximate time of ovulation and the approximate end of the follicular phase in ovulating animals). This difficulty was realized by Allen (1927) in his original formulation of the oestrin-withdrawal theory of menstruation, and both then and later (1932) he suggested that the level of oestrogenic stimulation does not fall until some time after ovulation, and that the secretion of oestrin begun by the ovarian follicles is continued by the corpus luteum. Observational data, mainly relating to the sexual skin, suggested a modification of this view (Zuckerman 1930), to the effect that the degree of oestrogenic stimulation falls abruptly about the middle of the cycle to a low level where it is maintained until menstruation occurs, The experiments reported in this paper fully justify this interpretation. for it has been shown that when the oestrone level falls to some point within a fairly fixed threshold range bleeding is delayed until about the 28th day (fig. 5). Above that range bleeding does not occur (fig. 4), and below it bleeding is apparently not significantly retarded (see fig. 3).

The mid-cycle fall in the degree of oestrogenic stimulation is clearly not dependent on the occurrence of ovulation, since it takes place in anovular cycles as well as in cycles in which ovulation occurs. Moreover, the fall must be relatively considerable, since the sexual skin, which is very sensitive to oestrogenic stimulation, is inactive in the second half of the cycle. In normal rhesus monkeys it responds readily to the injection of oestrone in this part of the cycle (unpublished data). The pronounced mid-cycle diminution in the degree of oestrogenic stimulation is believed to be dependent on a corresponding fluctuation in the degree of gonadotropic stimulation.

The evidence presented in this and in the preceding paper shows that quite apart from the question of the amount of hormone available, the relative effectivity of oestrogenic stimulation may decrease in consequence of a rise in the oestrone threshold. The changes in the threshold appear to occur in a rhythmical manner. In the immature animal (OM. 78) in which they were best observed they recurred at intervals of from five to seven weeks. It is likely, for the following reasons, that the cycles of threshold change are of shorter duration in mature animals.

In man both the range and the variability of the menstrual cycle decrease "subsequent to the experience of from 20 to 24 menstrual cycles. After the twenty-fourth cycle a definite progressive trend toward regularity is seen" (Engle and Shelesnyak 1934). In view of the close correspondence in length of the human and rhesus cycles (Zuckerman 1937*c*), it is very likely that a similar process of maturation occurs in subhuman Primates, and it is also likely that as the variability and range of the duration of the menstrual cycle decrease with age, the variability and range of the cyclical fluctuations of the oestrone threshold decrease, and that these cycles are brought into chronological correspondence with the menstrual cycle. This point is at present being investigated experimentally.

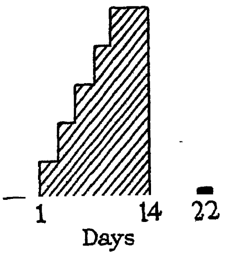

Fig 2

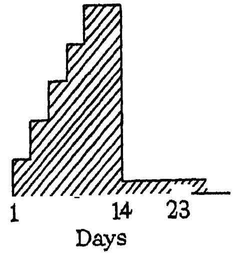

Fig.3

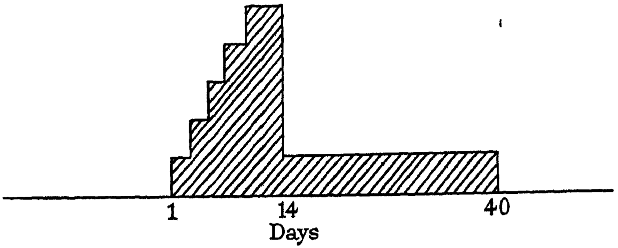

Fig.4

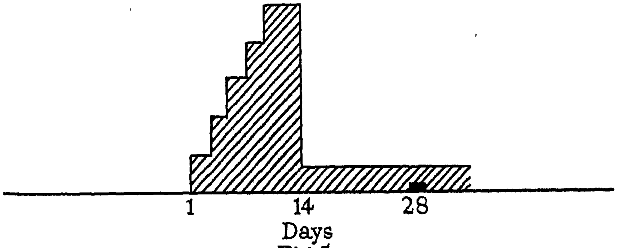

Fig.5

FIGS. 2–5—The areas marked in diagonal lines indicate the duration and degree of oestrogenic stimulation. The small black rectangular areas indicate phases of bleeding. The diagrams, which have not been drawn to scale, summarize the data given in Tables I–IV.

FIG. 2—The "artificial cycle" demarcated by the first of a 14-day series of injections and the first day of oestrin-withdrawal bleeding is 22 days.

FIG. 3—The daily administration after the end of such a course of injections of a very low dose of oestrone (50 I.U.) may not significantly delay the onset of bleeding. Artificial cycle = 23 days.

FIG. 4—Bleeding is completely inhibited if the dose from the 15th day onwards is too far above the threshold range (e.g. 400 I.U.).

FIG. 5—An artificial cycle of about 28 days is demarcated if the low level of oestrone from the 15th day onwards is within the upper part of the threshold range (e.g. between 100 and 150 I.U. daily).

Uterine bleeding will not occur in spayed monkeys during the course of daily injections of an amount of oestrone that is well above the range of fluctuation of the threshold (see for example, experiment OM. 98, preceding paper). Similarly menstrual bleeding can be inhibited in normal mature animals, presumably indefinitely, if a sufficient amount of oestrone is injected daily* (Zuckerman 1935, 1936*a*; Zondek 1936). This effect cannot be directly ascribed (as some have attempted to do) to any indirect action of oestrone on the anterior lobe of the pituitary, since even if the gonadotropic functions of that organ are disturbed, there is nevertheless sufficient oestrone within the animal to influence the uterus. This fact demonstrates the general validity of oestrone-withdrawal theories of menstruation.

The correspondence of the histological changes in the endometrium in normal anovular cycles and in the artificially stimulated endometrium is shown in figs. 6 and 7, Plates 17, 18. Fig. 6 is a photomicrograph of the endometrium on the first day of oestrin-withdrawal bleeding in a spayed rhesus monkey. Fig. 7 is a photograph of the endometrium on the first day of bleeding at the end of a normal anovular cycle in a Talapoin monkey, *Cercopithecus talapoin*. The condition of the glands and stroma is the same in both specimens. In view of the general considerations outlined above, and since the "artificial cycles" simulate normal anovular cycles both in regard to duration and in regard to the changes that occur in the endometrium, it may be concluded that the manner in which the endometrium is controlled by oestrogenic hormone in normal cycles is essentially the same as that by which the "artificial cycles" were controlled in the present series of experiments.

Three general objections have been raised against the view that menstrual bleeding may be a phenomenon related to the cessation of oestrogenic stimulation. The first is that bleeding sometimes occurs during the course of oestrone injections. This objection has been widely but uncritically accepted, but as shown elsewhere (Zuckerman 1936*a*) the evidence relates to the bleeding that occurs during the course of injection of threshold doses, and the objection does not apply. The second objection against the oestrin-withdrawal theory relates to the times of concentration of oestrogenic substances in the blood and urine. The peaks of concentration occur just after the time of ovulation and just before menstruation sets in (Frank 1935; Fluhmann 1936), and it has been widely argued that the fact that oestrogenic substances are present in high concentration in the body fluids just before menstruation occurs indicates that bleeding cannot be due to any lack of oestrogenic stimulation. The significant fact that has been overlooked in this argument is that little or no oestrogenic substance can be detected in the body fluids at the time oestrin produces its positive morphological effects (i.e. in the first half of the cycle). In view of this and other considerations it was suggested (Krohn and Zuckerman 1937) that the presence of oestrogenic substance in the blood stream during the menstrual cycle is perhaps indicative of its non-effectivity and of its imminent excretion. The first peak of concentration of oestrogenic substances in the body fluids coincides with the first depression of the level of oestrogenic stimulation (as indicated by the subsidence of the sexual skin). The second, which occurs just before menstruation, probably coincides with a relative decrease in the level of oestrogenic stimulation due to a rise in the oestrin threshold of the body. A further correlation is possible. By examination of the total solids in the uterus of monkeys at different times of the cycle van Dyke and Ch'en (1936) have found that the water content of the endometrium falls after ovulation and that the uterus is further dehydrated at the time of menstruation. A considerable process of dehydration also occurs in the sexual skin at the time of the mid-cycle fall in the level of oestrogenic stimulation (time of ovulation) (Krohn and Zuckerman 1937). The dehydration of the uterus at the time of menstruation may therefore be related to the fall, which has been postulated above, in the level of oestrogenic stimulation at the end of the cycle. The terms of this argument may be stated briefly as follows. When the mid-interval fall in the level of oestrogenic stimulation occurs (as indicated by the subsidence of the sexual skin) maximal dehydration of the subcutaneous tissues and partial dehydration of the uterus take place. At the same time the concentration of oestrogenic substances in the blood rises. When the concentration of oestrogenic substances in the blood stream rises again at the end of the cycle, further dehydration of the uterus takes place. The obvious inference that the degree of oestrogenic stimulation also falls at the end of the cycle is supported by the finding that the oestrone threshold of the body fluctuates cyclically and that bleeding is more likely to occur in the second half of the cycle when the threshold is high.

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* If injections are started in the second half of ovulatory cycles the ensuing phase of bleeding may not be inhibited (Zuckerman 1935; Corner 1935). The failure of oestrin to inhibit bleeding in such cases is presumably due to the influence of the corpus luteum.

The third objection raised against oestrin-withdrawal theories of menstruation concerns the corpus luteum. This objection holds only for cycles in which ovulation occurs. It has been shown (Engle, Smith and Shelesnyak 1935) that the administration of progestin at the end of a course of oestrin injections delays oestrin-withdrawal bleeding, and Corner

(1935) has found that progestin can inhibit normal menstrual bleeding.[*] Experiments of my own, the details of which will be given later,[†] show clearly that a quantitative relation exists between oestrone and progesterone in the control of uterine bleeding. When administered simultaneously, the two may undoubtedly hinder each other's action, and it is not unlikely that in normal cycles in which ovulation occurs the luteal secretions constitute an accessory mechanism whereby the level of oestrogenic stimulation is depressed. Thus the administration of progesterone in very high doses for a period during the course of continuous daily injections of an amount of oestrone that is well above threshold value may result in the precipitation of uterine bleeding after the cessation of the progesterone injections and in spite of the injections of oestrone. Similarly, an amount of progesterone in excess of that necessary at other times to transform an endometrium from the "interval" (fig. 1, Plate 17) to the "progestational" type (fig. 8, Plate 18) may be rendered ineffective by the simultaneous administration of a high dose of oestrone. The two hormones are almost certainly perfectly balanced against each other in the luteal phases of cycles in which ovulation occurs, but the part the luteal hormone plays can only be secondary to that of the oestrogenic hormone, for uterine bleeding takes place at regular "menstrual" intervals whether or not a corpus luteum is formed. The influence of the corpus luteum is that of a powerful controlling agent. If the corpus luteum failed to degenerate, as it normally does, towards the end of a menstrual cycle (e.g. as happens when fertilization has occurred), and sufficient luteal stimulation of the endometrium continued, uterine bleeding would not take place. These questions will be considered further in the concluding part of this series.

I wish to express my best thanks to Professor W. E. Le Gros Clark, F.R.S., for providing the general facilities that allowed of this study and to Dr. A. S. Parkes, F.R.S., for his critical interest in the work. I wish also to thank Mr. P. L. Krohn, Mr. A. N. Guthkelch and Dr. A. E. Russell for assisting at various times in the giving of injections. The oestrone was very kindly provided by Dr. Parkes and by the Organon Company. The monkeys were bought with the aid of a grant from the Medical Research Council.

[*] In some recent experiments I have found that testosterone propionate, whose properties overlap those of progesterone to a certain extent, has the same effect.

[†] Preliminary notes on these experiments have been published (Zuckerman 1936*b*, 1937*a*).

SUMMARY

Spayed rhesus monkeys were injected with increasing amounts of oestrone for 14 days, the maximum daily dose ranging in different experiments from 400 to 5000 I.U. On the 15th day the dosage was reduced to a constant level ranging from 50 to 300 I.U. If bleeding occurred during the course of the low level of injections the experiment ended and a new one was begun. The period between and including the first day of injections and the first day of bleeding constituted an "artificial cycle". If bleeding did not take place, injections were discontinued on the 40th day, and a new experiment was begun after the occurrence of oestrin-withdrawal bleeding.

The mean length of thirty-nine "artificial cycles" that occurred in sixty-two such experiments was 26·77 days. The mean length of "control cycles" that comprised the interval between bleeding and the first day of equivalent 14-day periods of injection was 22·31 days. The difference between the two means is 4·46 ± 0·66 and is clearly significant.

The extent of the 15th-day fall in the level of oestrogenic stimulation appears to have no effect on the occurrence of bleeding. Bleeding occurs sooner when small amounts of oestrone are given during the first 14 days and when the level to which the dose is dropped on the 15th day is in the lower part of the threshold range.

Those animals whose previously tested oestrone thresholds were high never bled (except in two of twenty cases) on doses higher than their thresholds. Those animals whose tested thresholds were low (50 or 25 I.U.) bled in sixteen of eighteen cases on doses which were from two to five times their tested thresholds. It is concluded therefore that the threshold fluctuates over a wide range (from about 50 to 250 I.U.), and that some animals had been tested at the bottom and others at the top of a curve of oestrone sensitivity. This conclusion is related to the finding, reported in the previous paper, that cyclical fluctuations occur in oestrone threshold.

These findings are discussed in relation to a theory of the mechanism of the menstrual cycle.

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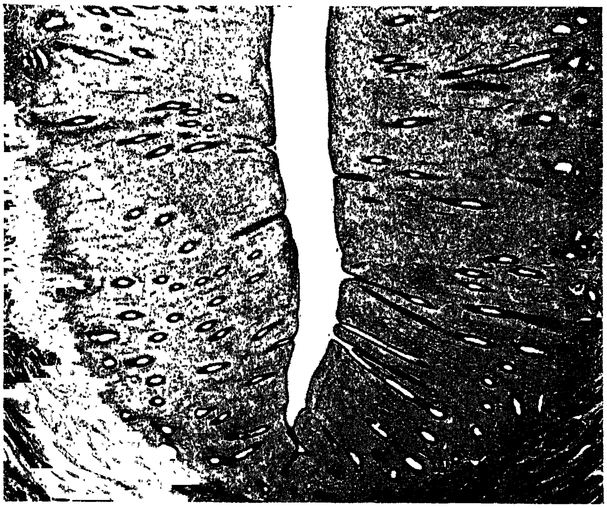

FIG. 1

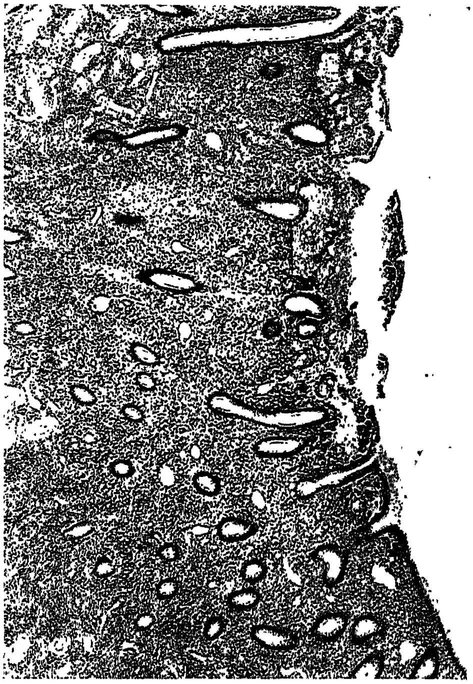

FIG. 6

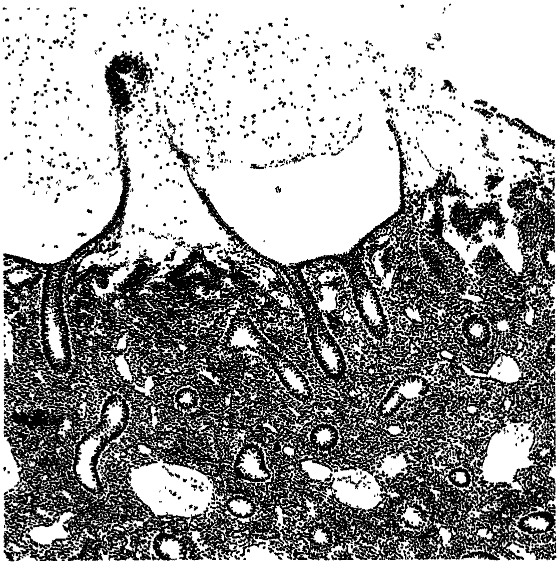

Fig. 7

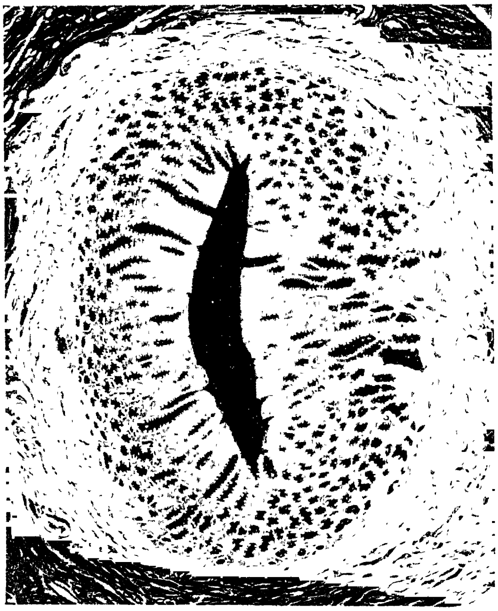

Fig. 8

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DESCRIPTION OF PLATES

Plate 17

FIG. 1—Endometrium of OM. 96 after fourteen daily injections of oestrone. Total injected 2·7 mg. × 10.

FIG. 6—Endometrium of OM. 95 on first day of oestrin-withdrawal bleeding, showing regions of extravasation and breakdown in the superficial zone of the endometrium. × 54.

Plate 18

FIG. 7—Endometrium of normal Talapoin monkey, *Cercopithecus talapoin* (M. 54) on the first day of bleeding at the end of a cycle in which ovulation did not occur. × 60.

FIG. 8—Progestational endometrium in OM. 106, following injections of progestin. The animal had first undergone a course of oestrone injections. × 5½.

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The Mechanism of the Lateral Sense Organs of Fishes

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[Plates 19–22]

Introduction

For a long time after their discovery in the seventeenth century the lateral-line canals of fishes were considered to be mucus-secreting organs. In 1850 Leydig described sense organs in the lateral-line canals, and this discovery stimulated a keen interest in the investigation of both the morphological and functional features of the lateral-line system. Morphological studies have yielded a thorough understanding of the structure of these organs (Ewart and Mitchell 1892; Cole 1896; Johnson 1917; von Woellwarth 1933). Physiological studies, though numerous, have been less fruitful. An account of the older work was given by Baglioni (1913), and the more recent work is reviewed by Dykgraaf (1933). The only technique until recently available has been the elimination of the sensory system by nerve section and cauterization, and the comparison of the behaviour of intact and operated fishes in response to various stimuli. With so diffuse a structure as the lateral-line system, receiving its nerve supply from the fifth, seventh, ninth and tenth cranial nerves, this method is particularly inadequate, and involves a violent mutilation of the animal. When one considers the crudity of many of these operations, it is not the uncertainty of the results which is remarkable, but rather that some of the conclusions reached should remain valid to-day in the light of far more penetrating experimental analysis.

This method of organ elimination could yield at best only an indication of the kind of stimulus that is effective in evoking the excitation of lateral-line receptors. In current textbooks the conclusion of Parker (1904) that the effective stimulus for the lateral line is low-frequency vibration, and that of Hofer (1907) that it is movement of water (i.e. local currents) have received most notice. The observations of Dykgraaf (1933), who employed the more refined methods of von Frisch's *futterdressur* technique, support Hofer's conclusion, and to some extent also Parker's. Dykgraaf considers the lateral-line system to be an organ of *Ferntastsinn*, and if this is taken to mean a mechanoreceptor of such sensitivity that it can function both as a touch organ and as a receptor for disturbances coming from a distance, it is undoubtedly a true description, for it is fully confirmed by the direct electrophysiological studies of Hoagland (1933 *a*, *b*, *c* and *d*) and of Schriever (1935). The latter, apparently unacquainted with Hoagland's work, did little more than to confirm several of his observations.

Hoagland discovered the spontaneous activity of the lateral-line receptors. Using an amplifier and Matthews' oscillograph, he showed that there is a persistent discharge of action potentials in the lateral-line nerve, whose frequency is a function of the number of neuromasts with which the nerve remains in physiological connexion. The receptors can be stimulated mechanically by stroking or pressing on the skin over the lateral line, or by means of weak currents of water directed against it, and also by the passive flexing of the trunk as in swimming. The impulse discharges evoked by such stimulation are superimposed on the background of spontaneous activity in the nerve. Tuning forks of 100–250 c./sec. applied to the side of the vessel containing the preparation also had an effect on the receptor apparatus, though the response did not reproduce the frequency of the stimulus, but appeared as a synchronization of the spontaneous activity into a rhythm of beats at a frequency of 20–70/sec. No responses of individual receptor units to tuning-fork vibrations were recorded.

When preparations containing one or two active units were subjected to different temperatures, it was found that the frequency of discharge increased exponentially with the temperature.

The oscillographic technique, in addition to providing a direct method for the investigation of the kinds of stimuli that are effective for different sensory structures, has been uniquely successful in advancing the analysis of the *mechanism* of sensory activity. In reviewing his observations on the lateral line in a monograph, Hoagland (1935) makes the first attempt to discuss the mechanism underlying the diversified responses which his experiments have disclosed. Here difficulties are encountered. The main difficulty is that responses to touch, pressure and bending of the trunk are all rapidly adapting, and rapid adaptation is a property which cannot be reconciled with the vigorous spontaneous activity which is so characteristic a feature of lateral-line receptors. The distinction between rapid and slow adaptation is a fundamental one, as Adrian (1928) has pointed out.

It is inconceivable that a tactile ending which adapts rapidly to constant touch and pressure should generate a persistent discharge of impulses except under the influence of repetitive stimulation as in the experiment of Adrian, Cattell and Hoagland (1931) on the frog's skin stimulated by means of an interrupted air blast. To overcome this difficulty Hoagland suggests that there are two kinds of receptors in the lateral-line system. His conclusions are best stated in his own words: "...there are at least two groups of lateral-line receptors, one directly responsive to tactile and pressure stimuli and to compression from surrounding tissues, giving impulses only when so stimulated and showing rapid adaptation to a constant stimulus; the other continuously active, emitting impulses repetitively at frequencies determined by a continuous process in the receptors. These organs apparently mediate responses of the fish to temperature changes in the water, and at the same time may act as very sensitive mechanoreceptors by means of their hair cell protuberances" (1935, p. 70).

This functional dichotomy has, in Hoagland's view, a morphological basis. "One group, the so-called pit organs, are rapidly adapting tactile receptors of considerable sensitivity, the other group, the spontaneously active neuromasts, show no adaptation at all..." (p. 83).

The distinction between pit organs and canal neuromasts is purely conjectural, since it was not found possible to isolate either group. Indeed, the distinction rests on the observation that the impulses evoked by mechanical stimulation appear to be conducted in fibres not concerned in the spontaneous discharge. Moreover, it is difficult to understand how the spontaneously active neuromasts can act as "very sensitive mechanoreceptors" if they are unaffected by touch and pressure. Their remarkable spontaneous activity appears to have no special significance, unless, as Hoagland suggests, they are to be regarded as "having some sort of tonic reinforcing function in the central nervous system" (p. 52). If this is taken to imply a proprioceptive function, it has to be admitted that in this respect the rapidly adapting receptors (pit organs) belong to the same functional system, for Hoagland ascribes to them the impulse discharges produced by bending of the body, which "may serve proprioceptively in the control of swimming movements" (p. 51).

If one takes into consideration the special structural features of the lateral-line canal system one can advance a hypothesis which avoids the apparent inconsistencies of Hoagland's interpretation. The lateral-line receptors are secondary sense cells with hair-like processes projecting into canals containing a fluid. Their excitation, it is generally supposed, is produced by a displacement of the sense hairs. Such displacement would be caused in the first place by a movement of the fluid in the canal, and in order to maintain a permanent deformation of a sense hair the fluid would have to flow steadily at a constant rate. Mechanically, the sense hairs may be compared to reeds in a river bed. They remain bent while the stream flows, but if the current should cease their elasticity would bring them back to the vertical position against the resistance of the fluid medium. The effect of pressure on the skin of a fish is to cause a movement of the fluid in a canal of the lateral line at the moment when the stimulus is applied, but once the rising phase of pressure has been accomplished and the pressure reaches a constant level of intensity the fluid in the canal comes to rest again. This is easily seen under a binocular in a canal injected with fluid containing particles in suspension when light objects are allowed to rest on the skin near the canal.

It is evident, therefore, that touch and pressure on the skin do not provide a "constant" stimulus so far as the sense hairs are concerned. Their deformation is transient, and the brief discharges of impulses which occur when the skin is touched or pressed cannot be taken as evidence of rapid adaptation. To study the adaptation of lateral-line receptors it is necessary to produce a permanent displacement of the sense hairs by means of a steady flow of fluid along the lateral-line canal.

The lateral-line canals of teleostean fishes are delicate structures, and it is not practicable to achieve a controlled movement of fluid in them. But in elasmobranchs the canals lie embedded in thick skin, and their diameter is sufficiently great to permit the insertion of a fine seeker. In the ray it has been found possible, after many trials and failures, to cannulate a portion of a lateral-line canal and to perfuse it with fluid at constant pressure. Once one has carried out such a perfusion and has observed its effect on the discharge of impulses in the nerve supplying the canal, one is no longer in doubt that here are the true conditions for constant stimulation of lateral-line receptors, and that all other mechanical stimuli, such as touch, pressure and even vibrations set up at a distance, are effective only inasmuch as they can produce a transient movement of the fluid in the canal.

Hoagland's distinction between rapidly adapting and spontaneously active receptors then disappears, and we are left with a single sensory mechanism which, although entirely unlike familiar types of mechanical receptors, is adequate to account for all the responses to mechanical stimuli that have been observed, and which, moreover, furnishes an intelligible functional interpretation of the morphological and ontogenetic characteristics of the lateral-line system.

EXPERIMENTAL RESULTS

General Observations—Experiments have been performed on over one hundred specimens of fresh-water and marine fishes. The fishes used were the roach (*Rutilus rutilus*), the rudd (*Scardinius erythrophthalmus*), the plaice (*Pleuronectes platessa*), the dogfish (*Scyllium canicula*) and the rays (*Raja clavata, maculata* and *microcellata*). Action currents in the lateral-line nerves, and also in the superficial ophthalmic and hyomandibular nerves of the rays, were recorded in the usual way with a condenser-coupled five-stage amplifier, Matthews's oscillograph and loud-speaker.

In all these fishes Hoagland's main observations were confirmed. Their lateral-line nerves are constantly active, and records from whole nerves show a strong complex asynchronous discharge. Touch, pressure, jets of water, stirring of the water near the fish and flexing of the body all excite discharges which appear in the records as regions of intensified oscillation of the light beam from the oscillograph. The results were the same whether the fish was immersed in water or lay on a board in air. They were the same in decapitate preparations, chronic spinal animals, pithed preparations and isolated segments of body wall or fin (in rays) containing short lengths of canal and nerve. It is interesting to note, however, that in some early experiments when urethane anaesthesia was employed, the spontaneous discharge was frequently weak or absent.

The mechanical sensitivity of good preparations is remarkable. The gentlest tapping on the table on which the preparation is set up is sufficient to evoke responses. Even footsteps on the floor of the laboratory stimulated the preparation until this embarrassing source of interference was eliminated by mounting the table on thick pads of felt.

To provide a constant source of vibrational shocks a simple device was employed. A small rubber stopper fixed to the end of a glass rod 24 cm. long was mounted in a Palmer straw holder so as to form a light hammer which was driven by clockwork and made to fall through a distance of 8 cm. on to a rubber sponge lying on the edge of the table on which the preparation was set up. The rate of tapping was regulated to about 2/sec. The sponge lay at a distance of about 3 ft. from the preparation. The shocks imparted to the table by the tapping of the hammer were quite imperceptible to human touch. They could, however, be perceived as a slight tremor on the surface of the water in which the preparation was immersed. Responses to such hammer tapping are shown in fig. 1 (Plate 19).

Tests with a vibrating tuning fork did not yield clear results. Touching the table with the tuning fork produced some response whether the fork was vibrating or not, though there was some indication that the vibrating fork was the more effective, but one could not be sure of this. In several very sensitive preparations a clear response could be evoked by holding the tines of the vibrating fork about 1 cm. above the lateral-line canal, and two preparations responded to the humming of low tones at a distance of 2 or 3 ft. Records of this response showed a synchronization of the irregular spontaneous discharge into a rhythm of beats at about 15/sec., but the response to the tuning fork showed no such synchronization.

Both the spontaneous activity and the mechanical sensitivity varied within wide limits in different preparations and at different times. In freshly killed preparations with the whole nerve laid across the electrodes the spontaneous discharge is always vigorous and the vibrational sensitivity to gentle tapping strong. If the preparation is well irrigated, but otherwise undisturbed, this state of affairs may persist for several hours. But usually the preparations were manipulated in various ways. The nerve was cut down so as to leave only a few active units, the lateral-line canal was cut across, cannulated and perfused at various pressures. With such treatment the spontaneous activity declined more rapidly, and eventually would disappear altogether, leaving the preparation still sensitive to vibration and all other types of mechanical stimulation (fig. 1, Plate 19). At other times the vibrational sensitivity also vanished, and the preparation would then respond only to direct touch, as well as to the perfusion of the canal. In several experiments both the spontaneous activity and the vibrational sensitivity, having been absent for an hour or so, came back again at full strength. The causes of this interesting effect can only be surmised, but there is some indication that the maintenance of the spontaneous discharge depends upon a certain minimal hydrostatic pressure in the canal.

Perfusion Technique—Fig. 2 (Plate 19) is a photograph of the ventral surface of a ray whose lateral-line canals have been injected with indian ink. The ease with which the ink runs round once the hypodermic needle has been correctly inserted is a clear indication of the non-viscous character of the endolymph contained in the canals. In *Raja batis*, Ewart and Mitchell (1892) figure quite a large number of pores on the ventral surface, but in this ray, *R. maculata*, and indeed in all the rays that I have examined, there appears to be only one on each side, at the end of the long tubule at *P*. The hyomandibular loop therefore provides a long stretch of canal which has no openings to the exterior, and, when cannulae are successfully inserted, forms a closed system through which fluid can be made to circulate at a controlled rate. To achieve this requires considerable practice, because, of course, the canal is barely visible in the fresh preparation, and there is little to indicate its exact position. Two small glass tubes, about 1 mm. diameter, were tied into the canal—one, anteriorly directed, at *A*, the other, posteriorly directed, at *B*. In order successfully to cannulate the canal it was necessary to select large rays, and it was therefore convenient to isolate the portion of the pectoral fin containing the hyomandibular loop. Such isolated preparations usually remained active for 6–8 hr.

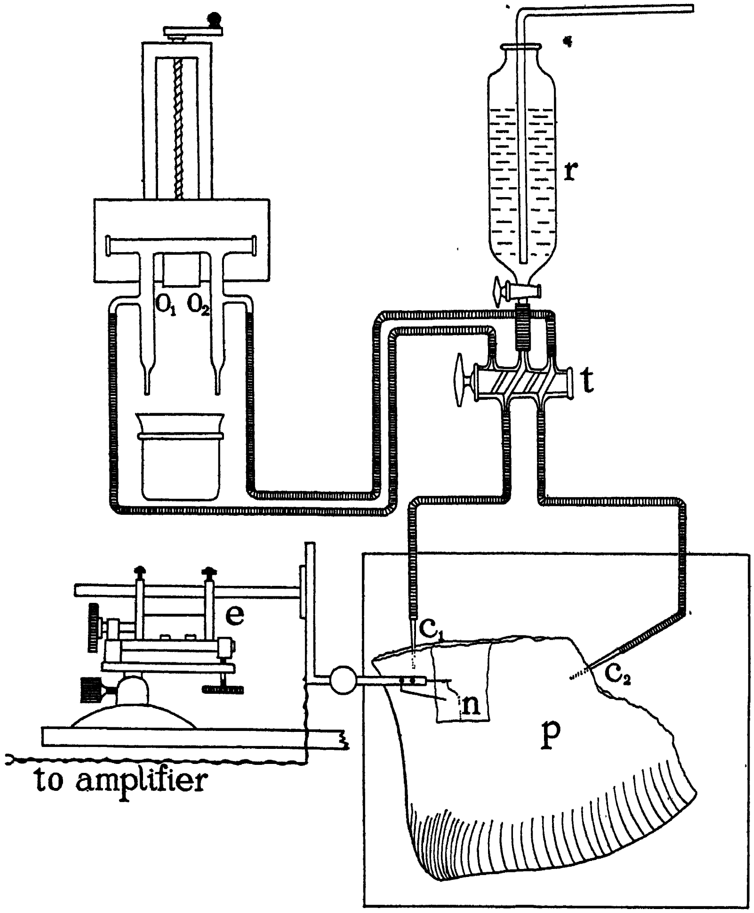

FIG. 3—Arrangement for perfusing the hyomandibular canal at a controlled rate. c_1, c_2, cannulae; *e*, electrode holder; *n*, peripheral branch of hyomandibular nerve; o_1, o_2, overflow levels; *p*, pectoral fin of ray; *r*, reservoir; *t*, five-way tap.

The arrangement of the perfusion system is shown in fig. 3. The cannulae were connected by rubber tubing to a five-way tap, which made connexion with a reservoir, and with two overflow tubes mounted together on the carriage of a Palmer screw stand. The reservoir was filled with dilute sea water containing urea (Young 1933), which is a satisfactory physiological fluid for elasmobranch tissues. The level of fluid in the reservoir was kept constant by an inverted syphon dipping into a large jar containing the same fluid (not shown in fig. 3). Turning the tap through 180° reversed the flow of fluid through the canal, and, of course, in the half-way (horizontal) position of the tap the portion of the circuit containing the canal was closed, and no fluid flowed. By raising or lowering the outflow levels, the head of water could be quantitatively controlled, and the rate of flow could be determined by counting the drops at the outflow with a stop-watch. Fig. 4 illustrates the accurate working of the perfusion system. The experiments were performed at a temperature of 12–14° C.

The hyomandibular loop is innervated by the hyomandibular branch of the facial nerve, which runs backward between the inner limb of the loop and the gill slits, and sends long slender branches beneath the skin to every portion of the canal. These branches can be found and dissected. They contain mostly fifteen to thirty fibres. The nerve, having been dissected under a binocular, ligatured and cut centrally, was slung on a silver wire electrode mounted on a micromanipulator (shown in fig. 3). This electrode was connected to the grid of the first valve of the amplifier, the earth electrode was inserted into the muscle close by. The usual precautions were observed to insure adequate electromagnetic screening. The sensitivity of the recording system was of the order of 4 mm. deflexion for 20 μV across the input.

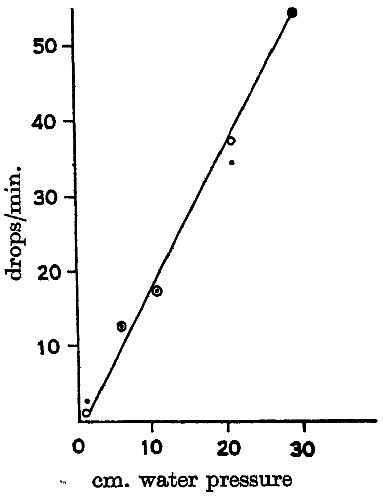

FIG. 4—Graph showing the rate of flow of fluid through the hyomandibular canal loop at varying pressures of water. Dots, headward flow. Circles, tailward flow.

Perfusion Experiments—Preparations cannulated and installed as described showed a vigorous complex discharge of action potentials. Perfusion of the canal evoked a strong response whose adaptation was very slow even when perfusion was continued for 60 sec. Touching and stroking the canal, rubbing or pinching the rubber tubes connected to the preparation produced brief but vigorous responses, and in most experiments vibrational shocks imparted to the table with the rubber hammer called forth volleys which were unmistakable both in the oscillographic records and in the rumble from the loud-speaker. The effects of perfusing the canal in the headward and tailward direction were usually not strikingly different when a large number of fibres was in action, and the impression at first gained from a number of experiments on whole nerves was that the receptors responded equally to a flow of fluid in either direction. More extensive experience showed, however, that this impression was false. It has already been indicated that with time, and especially after numerous perfusions, the spontaneous discharge declined, and eventually ceased. This process involved the gradual dropping out of spontaneously active units, the preparation becoming progressively simpler, and the records correspondingly less crowded. Records taken from such "attenuated" preparations showed a clear difference in the behaviour of the end-organs with respect to headward and tailward perfusion.

Fig. 5 (Plate 20) shows the responses to perfusion of a preparation from a ray killed at 12.45 p.m. The records were taken at the following times: A, 3.15; B, 3.20; C, 3.20; D, 4.15; E, 4.16. It can be seen that the response to headward perfusion (record B) is noticeably stronger than the response to tailward perfusion at the same pressure (record A). Moreover, the after-effects of the two perfusions are different. The spontaneous discharge in A, before the beginning of tailward perfusion, is accelerated as a result of that perfusion, as can be seen by comparing it with the spontaneous discharge in the first half of B. After 10 sec. of headward perfusion, however, the spontaneous discharge emerges much reduced (B and C), and only one unit appears to remain in action. About an hour later (record D) this unit is still firing, though at a reduced frequency, and, at the end of another 60 sec. of headward perfusion (record E) emerges unchanged.

It is clear from fig. 5 (Plate 20) that the behaviour of the end-organs is different both during and after perfusion with respect to the two directions of flow. In order to obtain further information on the nature of this difference it is necessary to reduce the number of active units by cutting down the nerve. This has been accomplished in many preparations, though attempts to isolate a *single* active unit have been successful on four occasions only.

Fig. 6 (Plate 21) shows records from a nerve that has been cut down in this way. In record A one unit is seen to be spontaneously active at a frequency of 5 or 6/sec., and there are some small random potentials as well. At the end of a headward perfusion continued for 60 sec. (record B) the large regular potentials have ceased, but the small potentials are discharging at an accelerated rate. After an interval of 1 min. rest (record C) the large potentials have appeared again, and the discharge of small potentials is less rapid. In record D, a tailward perfusion evokes a response which is strikingly weaker than was the response to headward perfusion in A, and which adapts considerably towards the end (E), and on the cessation of this perfusion there is actually an outburst of activity in which at least two units participate. Moreover, the small potentials discharging spontaneously in B, C and D are seen to accelerate during tailward perfusion (D and E), and to cease abruptly when this perfusion ends (E).

Fig. 7 (Plate 21) shows the behaviour of the same preparation 6 hr. after the fish was killed. Headward perfusion (record A) still evokes a strong response in which several units participate. During tailward perfusion (record B) the large potentials are inhibited, while the small potentials discharge at a greater rate.

The data of the experiments illustrated in figs. 5, 6 and 7 (Plates 20, 21), and several others of the same kind, indicate that in preparations in which a number of end-organs are active we have to deal with a fortuitous assortment of two antagonistic groups of receptors. Both groups possess a spontaneous rhythm of discharge. Their behaviour is modified by perfusion in the ways shown in the following table:

	Group 1		Group 2	
	During perfusion	After effect	During perfusion	After effect
Headward	Strong response	Silent period	Inhibition	Accelerated rhythm
Tailward	Inhibition	Accelerated rhythm	Response	Silent period

Although this classification is supported by the records, there are two points of detail which are obscure. The group 2 receptors always give potentials which are small in comparison with the group 1 type. The customary explanation of such a quantitative difference is that the smaller potentials are conducted in smaller fibres, but the question as to why the group 2 receptors should always appear to be connected with smaller fibres remains unanswered. If it be these units only which are excited by a tailward flow, then we must regard the large potentials recorded during perfusion in fig. 5A and fig. 6D (Plates 20, 21) as being due to a summation of such small potentials. Careful inspection of the records does not add conviction to such an interpretation. In particular, fig. 6 D (Plate 21 looks very much as though the spontaneously active large potentials are accelerated during the tailward perfusion, and yet 1½ hr. later the same preparation subjected to the same perfusion shows a clear inhibition of the large potentials (fig 7B, Plate 21).

These obscurities can be resolved only by an examination of isolated units. Of the four successful single-fibre preparations that have been achieved, three were of the group 1 type, and one was a group 2 unit.

Behaviour of a Single Receptor—Fig. 8 (Plate 22) illustrates the responses of a single-fibre preparation to perfusion in either direction. This was a large *Raja microcellata* in which the nerve was cut down soon after dissection, and, after a few trials, yielded a single unit firing spontaneously at a regular frequency. The figure shows the effects of a headward perfusion followed by a tailward perfusion after an interval of 2 min. The whole course of this experiment is illustrated graphically in fig. 9, in which the number of impulses in each successive second is plotted against time. It is to be noted that there is very little adaptation during 10 sec. of headward flow, and that on stopping perfusion there follows a prolonged silent period before the spontaneous frequency is re-established. Tailward perfusion causes an instant inhibition, complete for 2 sec. and partial for the remaining 8 sec., and on closing the tap the rhythm accelerates and is maintained

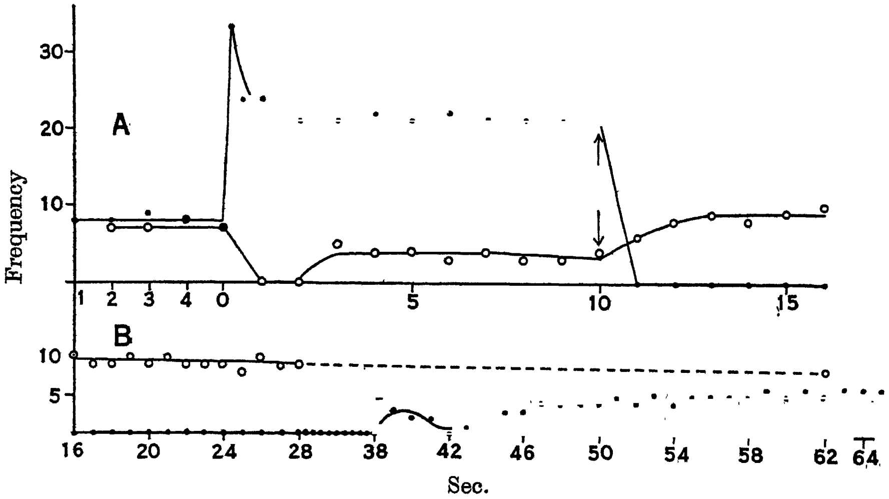

Fig. 9—Graph plotted from the records of the experiment of fig. 8. B is the continuation of A. Dots—headward perfusion. Circles—tailward perfusion. Perfusions begin at 0 sec., end at arrows. Rate of flow 12 mm./sec.

for many seconds at a frequency somewhat greater than the original spontaneous frequency.

That the stimulus is provided not by the pressure of water in the canal but by the actual flow of fluid is easily and convincingly demonstrated. When, for example, the canal is being perfused in a headward direction as in fig. 8 A (Plate 22), the discharge can be instantly stopped by clamping the *outflow* tube. On releasing the clamp the discharge starts up again. In fig. 10 (Plate 22) record A shows several fibres responding to headward perfusion, and record B shows the activity of the same preparation when the tap is turned on and off with the outflow clamped. The rise in pressure causes a momentary movement of the fluid in the canal owing to the elasticity of the skin and the distension of the canal wall, and this gives rise to several bursts of impulses, but once the new equilibrium is established no further discharge of impulses occurs. For this reason it is clearly more appropriate to express the perfusion rate in terms of flow in mm./sec., rather as cm. of water pressure. This has been done on the assumption that the diameter of the canal is 1·2 mm. Although an approximation, this value cannot be more than 25 % out, and the error which it introduces is constant for any one preparation. For the purpose of comparison, therefore, the figures are accurate, and even as absolute values they are useful. The rate of flow for the perfusions in fig. 9 works out at 12 mm./sec.

Figs. 11 and 12 both show the effects of perfusion at 5 mm./sec. on the same preparation. They illustrate the effects of the gradual decline of the excitatory state of the preparation. There was an interval of 10 min. between the two experiments, and during this interval headward and tailward perfusions of 10 sec. duration were performed. The initial spontaneous rhythm is slower in fig. 12 than in fig. 11, and the discharge evoked by headward perfusion is correspondingly less rapid. Tailward perfusion produces only a partial inhibition in fig. 11, whereas in fig. 12 the inhibition is complete throughout the duration of the perfusion. At the end of headward perfusion in fig. 12 the recording paper came to an end, but the usual silent period was clearly marked by the loud-speaker.

Fig. 13 is of great interest. The spontaneous activity had ceased, and the preparation had been quiet for 5 min. Headward perfusion gave the usual response, though the relatively strong stimulus of 20 mm./sec. flow evoked a frequency of discharge that was no greater than in the experiment of fig. 9, where the rate of flow was 12 mm./sec. After this perfusion there was again no activity in the nerve, and therefore, as was to be expected, perfusion in the tailward direction produced no effect. But on stopping this perfusion a regular discharge developed, and continued for over 5 min.

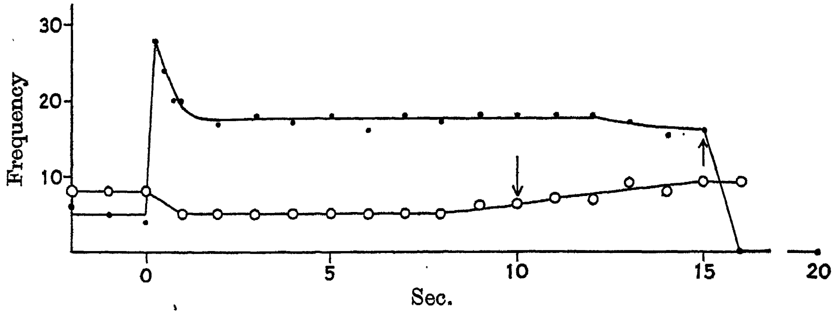

FIG. 11—Tailward perfusion followed by headward perfusion after a 2 min. rest interval. Circles—tailward flow. Dots—headward flow. Perfusions begin a 0 sec., end at arrows. Rate of flow 5 mm./sec.

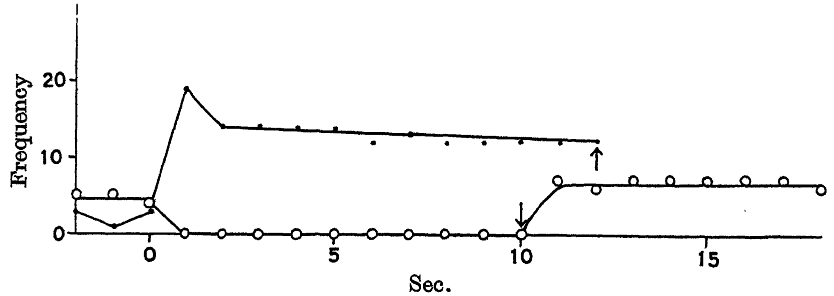

FIG. 12—Repetition of the experiment of fig. 11, when the spontaneous rhythm had declined by about 33 %.

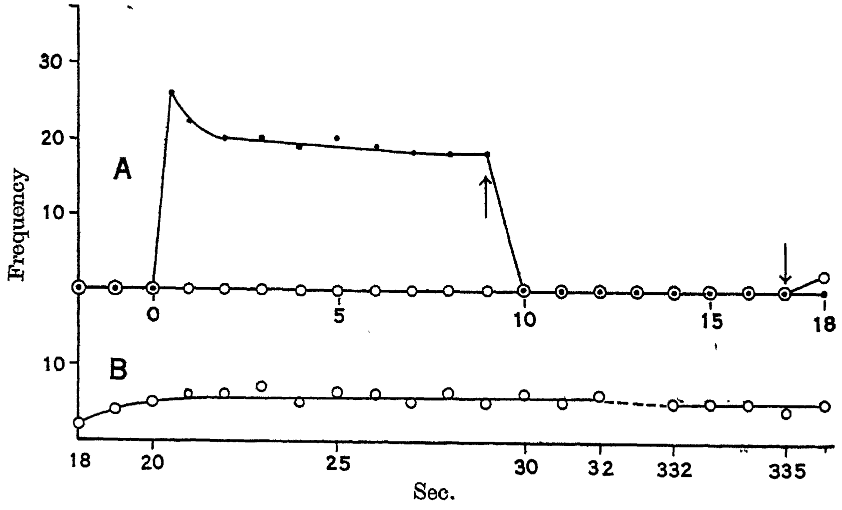

FIG. 13—Headward perfusion followed by a tailward perfusion after a 2 min. rest interval. Dots—headward flow. Circles—tailward flow. Perfusions begin at 0 sec., end at arrows. Rate of flow 20 mm./sec. B is the continuation of A.

This phenomenon provides a remarkably vivid demonstration of the way in which the tailward (inhibitory) flow modifies the excitatory state of the end-organ even though it appears to have no direct overt effect.

By way of control a perfusion was carried out with the overflow levels adjusted as near as possible to the height of the fluid in the reservoir. In view of the very great sensitivity of the preparation it was hardly to be expected that turning the tap on and off would be entirely without effect. Fig. 14 shows that, apart from a brief burst of five impulses lasting $\frac{1}{8}$ sec. when the tap was turned on as for tailward perfusion, the rhythmic activity of the receptor continued practically unchanged.

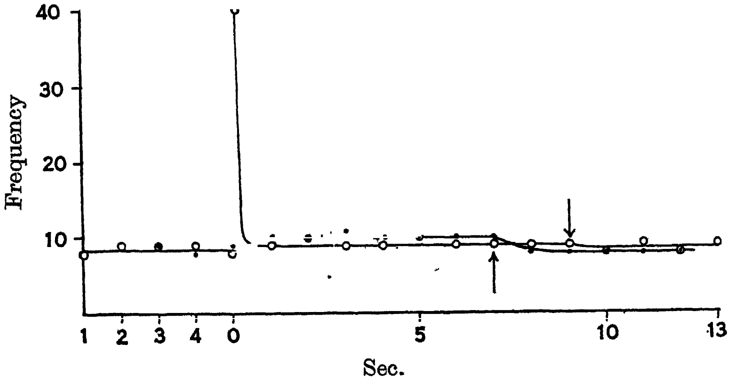

FIG. 14—Control "perfusions" at zero flow. Symbols as before.

The Quantitative Stimulus-Response Relationship—Weber's well-known law has been demonstrated in the muscle spindles of the frog (Matthews 1931), the stretch receptors of the ray (Fessard and Sand 1937) and the lateral eye of *Limulus* (Hartline and Graham 1932). It applies equally to the receptors of the lateral line. A series of twelve headward perfusions at different rates of flow were performed on a single fibre preparation which showed no spontaneous activity. Each perfusion was continued for 10 sec., and there were 1 min. intervals between successive perfusions, except between the sixth and seventh, when the preparation was rested for 30 min. The whole experiment therefore lasted 45 min. The rates of flow were varied in random order. The frequencies at 5 sec. after the beginning of perfusion are plotted on a large scale in fig. 15, and in fig. 16 the same data are plotted on a logarithmic scale. The determination of the rate of flow becomes very approximate at values below 2 mm./sec., and in fig. 16 the dotted line is just as likely to be true as the continuous line covering the same region of the curve. All that can be said is that the logarithmic relation holds for rates of flow between 2 and 16 mm./sec., and that it possibly holds for even lower rates of flow.

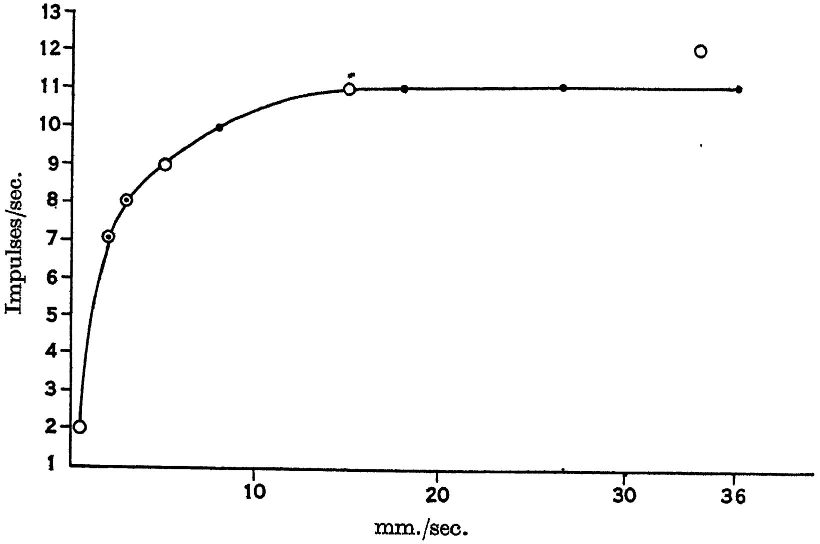

Fig. 15—Relation between the rate of headward flow and the frequency of discharge 5 sec. after the beginning of perfusion. Spontaneous rhythm was absent. Circles—determinations 1–6. Dots—determinations 7–12. Both sets in random order.

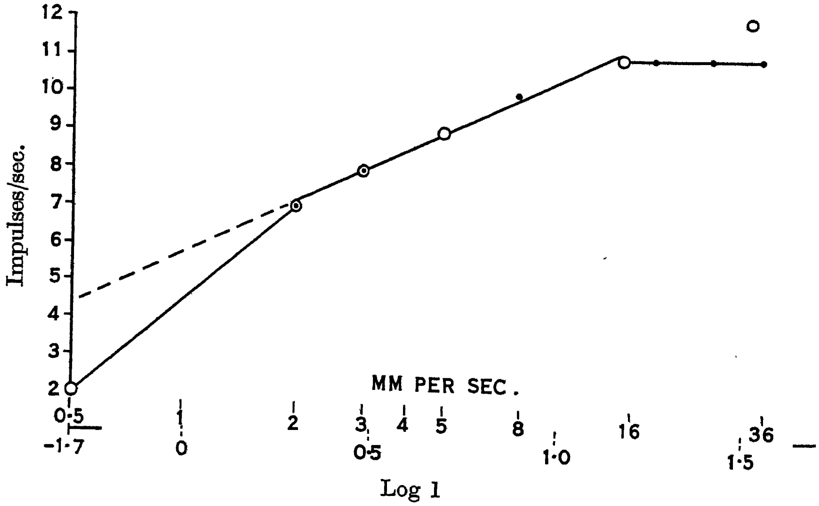

Fig. 16—The values of fig. 15 plotted on a logarithmic scale.

There are two points of interest that emerge from these determinations. The maximum frequency of discharge attained by the end-organ is remarkably low. It is true that this preparation was in a condition of relatively low excitability. It had been subjected to a good deal of manipulation before these determinations were carried out. By that time the spontaneous activity had ceased. But even in fresher and more vigorous preparations the receptor does not appear to be capable of discharging at a frequency greater than about 25/sec., except for very brief bursts of a few impulses, which may be somewhat more rapid. In keeping with this is the unusual thickness of the spikes as seen in figs. 6, 7 and 8 (Plates 21, 22), which, considering the slow camera speed, implies a fairly slow conduction rate. There has been no opportunity of determining the conduction rate in these fibres, though it certainly ought to be done. The second point to be noted is the low threshold of excitability. A rate of flow of about 16 mm./sec. appears to be a maximal stimulus for these receptors, and the threshold is so low that it could not be determined. It would be more correct to speak of a creeping rather than a flow of fluid as being the effective stimulus.

Effect of Perfusion on the Response to Vibration—In fig. 1 E (Plate 19) the burst of impulses evoked by the tapping of the hammer described on p. 476 are seen to be superimposed on the rhythmic discharge resulting from the perfusion of the canal. The sensitivity to vibration, however, is not independent of the flow of fluid in the canal. When a preparation is obtained which is sufficiently simple for the inhibition of the spontaneous rhythm during a tailward perfusion to be capable of demonstration, and which at the same time is sensitive to the vibrations set up by the tapping of the hammer, it is found that, like the spontaneous rhythm, the responses to tapping are modified or suppressed during perfusion depending on the rate of flow. Fig. 17 (Plate 20) shows records from a preparation in which a single fibre was discharging spontaneously at a rate of 7/sec. At the same time bursts of larger potentials were evoked by the regular tapping of the hammer, which implies that, in addition to the spontaneously active unit, the preparation contained several other functional receptors which were not spontaneously active. During a tailward perfusion at a rate of 50 mm./sec. both the spontaneous rhythm and the responses to the hammer taps ceased (record A), and when the perfusion was discontinued (record B) the responses to the hammer at once reappeared, and the spontaneous rhythm began again a few seconds later. A second perfusion, 10 min. later, at a slower rate of flow of 17·5 mm./sec. (record C) again inhibited the spontaneous rhythm, but failed to suppress the responses to the hammer, which continued throughout the perfusion (record D). Nevertheless, it can be seen that the character of these responses was modified during the perfusion, inasmuch as the time interval between the two volleys of each pair is different from the time intervals before and after perfusion.

Although the hammer was adjusted so as to deliver a slow series of single taps, each tap set up a damped oscillation of the table and preparation, which, when recorded with a microphone connected to the input of the amplifier, turned out to have a frequency of about 32/sec. In different preparations and at different times the responses to the hammer varied. Sometimes each tap evoked but a single volley, at other times two or more volleys in rapid succession, as in records A and B, or, quite commonly, three unequally spaced volleys, as in records C and E. These variations are to be ascribed to changes in sensitivity resulting from the effects of perfusion. It is particularly significant that in the experiment of fig. 17 (Plate 20) the sensitivity to the hammer taps as judged by the number of volleys in each response increased shortly after the cessation of the inhibitory perfusion (record B). This is in agreement with the slight but frequently observed acceleration of the spontaneous rhythm following a period of inhibition. (In record C the spontaneous frequency before perfusion is 7/sec., in record E, after perfusion it is 8. Compare also fig. 8 (Plate 22).)

So far as it is possible to estimate short time-intervals in these records, the volleys evoked by the hammer taps appear to have a period of 1/32 sec., i.e. the period of the oscillation produced by the tap. At the beginning of the experiment (record A) the preparation was responding to the first two oscillations of each hammer tap, and after the inhibitory perfusion it responded to three and sometimes four consecutive oscillations. In record C the volleys correspond to the first, second and sixth oscillations, but during the tailward perfusion (record D) the response changes to two volleys corresponding to the first and fourth oscillations. At the end of the perfusion (record E) the response returns again to the rhythm—first, second and sixth.

It would be idle, without more extensive quantitative data, to attempt an interpretation of the theoretical implications of these phenomena. It is sufficient here to record that, like the spontaneous rhythm, the excitability of the receptors with respect to low-frequency vibrations may be reduced or even completely suppressed by perfusion of the canal.

THE PHENOMENON OF SENSORY INHIBITION

In the foregoing description the term "inhibition" has been applied to the effect of tailward perfusion of the hyomandibular canal on a receptor showing a spontaneous rhythm of activity. This is admittedly an unorthodox use of the term, and its use without qualification to describe this new type of sensory effect may appear at first sight to be objectionable. It was employed almost without premeditation because when repeated reference is made to the reversible arrest of a persistent rhythm, this is the only term that appears to be appropriate. Nevertheless, it is necessary to examine the phenomenon in detail to see to what extent it corresponds to current conceptions of the inhibitory process.

The facts that have to be considered with regard to the behaviour of lateral-line receptors are simple and few.

1—The rhythmic activity of the receptor can be reversibly arrested or slowed down by a mechanical stimulus which is the antagonist of that which serves to excite the organ.

2—The measure of this effect depends upon

(*a*) the intensity of the stimulus (figs. 9 and 11),

(*b*) the state of excitation of the organ as revealed by the frequency of its rhythm (figs. 11 and 12).

3—When the state of excitation is such that no spontaneous rhythmic activity occurs, the same stimulus, while producing no overt change in the condition of the receptor during its application, may yet be followed, after the cessation of the stimulation, by a prolonged and sustained rhythmic activity (fig. 13).

This last effect appears to be identical with the phenomenon of post-inhibitory rebound, which, as Sherrington (1925) has said, "shows that stimulation of the inhibitory afferent produces an inhibitory state even in absence of an excitatory state".

Comparison with the behaviour of a motor neurone is most clear when one considers a motor centre which is tonically active. Adrian and Bronk (1929) have recorded a persistent discharge of 10–25/sec. in fibres of the anterior crural of decerebrate cat. Concerning the significance of this discharge they say, "we have no doubt that these persistent low frequency discharges are the cause of the 'tonic' contraction of decerebrate rigidity...". "The discharge could be inhibited by pinching the ipsilateral foot.... When movements of extension are produced by stimulation of the contralateral foot the frequency of the impulses rises fairly rapidly...."

In comparing these observations with the behaviour of the lateral-line receptors it is necessary to consider the probable causation of the spontaneous discharge in lateral-line nerves. Is it indeed an inherent property of the receptor to generate a regular train of impulses in its nerve fibre, or is there some mechanism external to the receptor which supplies it with a permanent source of mechanical energy? Current conceptions of sensory activity certainly predispose one in favour of the latter alternative. Hoagland (1935) has argued that the hair processes of the lateral sense cells are, in effect, cilia, and in support of this suggestion he cites Bowen's (1932) observations on the hair cells of the labyrinth of *Amiurus*. Bowen's account of the movements which he saw in isolated pieces of an ampullary crista is not sufficiently convincing to serve as a basis for an established fact. The movements were few, sporadic and irregular. His description does not exclude the possibility that they were due to currents, surface tension effects and the like in the saline under his cover-slip. Ciliary activity, when regular and persistent, is usually unmistakable. I have injected a hyomandibular canal with a suspension of particles of methylene blue and have observed these particles under a binocular for over an hour. Throughout that period they remained motionless, yet the nerve from the same portion of canal showed a vigorous persistent discharge.

On theoretical grounds it is difficult to conceive how a secondary sense cell can be at the same time a contractile unit. So far as I am aware, no such "dual-purpose" structure has yet been encountered in any animal. Moreover, if the hair cells were indeed ciliary structures, they would not help to explain the behaviour of the lateral-line apparatus. It has not been found that the rhythm of ciliary activity could be augmented or reduced by currents of water flowing in this or that direction across a ciliary epithelium. If it is thought that ciliary activity of the hair cells is in some way more intelligible than an inherent rhythmicity of a purely nervous peripheral mechanism, it is only because we are familiar with such autonomous peripheral effectors, while the conception of an unprovoked peripheral nervous rhythm is new.

It is concluded, therefore, that the spontaneous discharge of lateral-line receptors is not due to any external supply of mechanical energy. The neuromast organs are believed to resemble certain motor centres in that they are in a state of excitation which gives rise to a regular stream of impulses in the nerves arising from them. This excitatory state is subject to greater excitation or to inhibition by antagonistic mechanical stimuli. The stimuli are movements of fluid in the lateral-line canals, and one can only suppose that their effects are communicated to the receptor by virtue of the bending of the sense hairs in one or the other direction.

CONCLUSION

The morphology of lateral-line organs is closely similar to that of the semicircular canals of the labyrinth, and their embryological relationship has been demonstrated (Johnson 1917). To a structural and ontogenetic connexion can now be added a common mode of behaviour. The receptors of the horizontal semicircular canal of the dogfish have been shown by Löwenstein and Sand (1936) to possess a spontaneous activity which is subject to excitation and inhibition by ipsilateral and contralateral rotation. Although it was not found possible to isolate a single labyrinthine end-organ, and the data therefore were less quantitative than in the present work, there can be no doubt that the performance of the two receptor mechanisms is essentially the same. There are, however, certain obscurities. The only condition for constant external stimulation of the lateral-line receptors is a flow of fluid in the canal. Static pressure in the canal is without lasting effect. There is no possibility of a steady flow in the semicircular canals, which are closed, and it is difficult to interpret the mechanical conditions which underlie the steady excitation (and inhibition) which occurs during prolonged rotation. It may be pointed out, however, that rotation at a constant angular velocity has, of course, a centrifugal component, and it may be this which supplies the energy for the steady displacement of the sense hairs, or of the cupula in which they are embedded, and which thus evokes the constant excitation of the end-organs during rotation. If this is so, then a constant *rectilinear* velocity should be without effect on the labyrinthine receptors. No direct experimental evidence on this point exists, but it is significant that Gray (1937) has observed that "no response of any kind was detected when blinded fish were subjected to rectilinear acceleration along any one of the three main axes of the body".

The excitation and inhibition of the receptors of the horizontal ampulla are significant in connexion with certain reflex effects of unilateral extirpation (see Löwenstein and Sand 1936). Moreover, so far as can be judged, all the receptors of a single ampulla are excited by ipsilateral, and inhibited by contralateral rotation. The two opposite ampullae are therefore truly antagonistic. In the lateral line, on the other hand, there appears to be no such regularity. The indication is that antagonistic receptors are mixed in a random assortment within each group of sense cells, and one cannot at present perceive any special significance in their orientation. This significance, whatever it may be, is to be sought in the reflex responses to lateral-line excitation, and since such reflexes are still unknown, their discussion is obviously premature. It is, however, fairly certain that under natural conditions the lateral-line receptors never get an opportunity of responding to a stimulus that is at all constant and prolonged, and their remarkable powers of slow adaptation would seem therefore to "lodge with them useless". Perhaps it would be more correct to regard slow adaptation as a logical, though not necessarily useful, consequence of spontaneous activity.

All the preparations dealt with in this work responded strongly to touch and pressure of the lateral-line canal. There are no pit organs associated with the hyomandibular canal of the ray, and therefore Hoagland's suggestion that rapidly adapting responses to mechanical stimulation are due to the pit organs is disproved. The responses are certainly due to the same receptors as the ones which possess a spontaneous rhythm of activity and which show a slow adapting response to perfusion of the canal. The indications are that touch and pressure directly applied to the lateral line are enormously supramaximal stimuli for receptors that can respond to a feeble vibration propagated through a considerable distance. It cannot be assumed, of course, that the same receptors are concerned in the vibrational sensitivity as in the responses to more violent mechanical stimulation. In most preparations reducing the nerve down to a few active units abolished the vibrational sensitivity. But of the four single end-organ preparations that have been successful, one showed responses to tapping of the hammer. These are shown in fig. 18 (Plate 21) together with the response to perfusion. The identical magnitude of the potentials in the two records, and the absence of any indication that more than one end-organ is active, lends support to the view that a vibrational shock is effective inasmuch as it produces in the fluid contained in the canal a tremor which is adequate to give rise to a very brief excitation of the spontaneously active, slow adapting receptors.

It is indeed very curious that the lateral-line system, being, as it is, a sensory apparatus of great mechanical sensitivity, should be so superficially situated on the surface of the body, and unprotected by skeletal structures. Its disposition is such that it must inevitably be stimulated by casual contacts of the fish's body with solid objects, as well as by the muscular rhythm of swimming. I have had occasion to observe lateral-line receptors responding to the sporadic twitching of a few muscle fibres in an isolated fin of the ray, and the discharge produced in the lateral-line nerve of the dogfish and teleostean fishes when the body is flexed as in swimming is very strong indeed. So long as our knowledge of the physiology of the lateral line is confined to its sensory mechanism the problem of its role in the normal behaviour of fishes must remain unsolved. It is evident that it

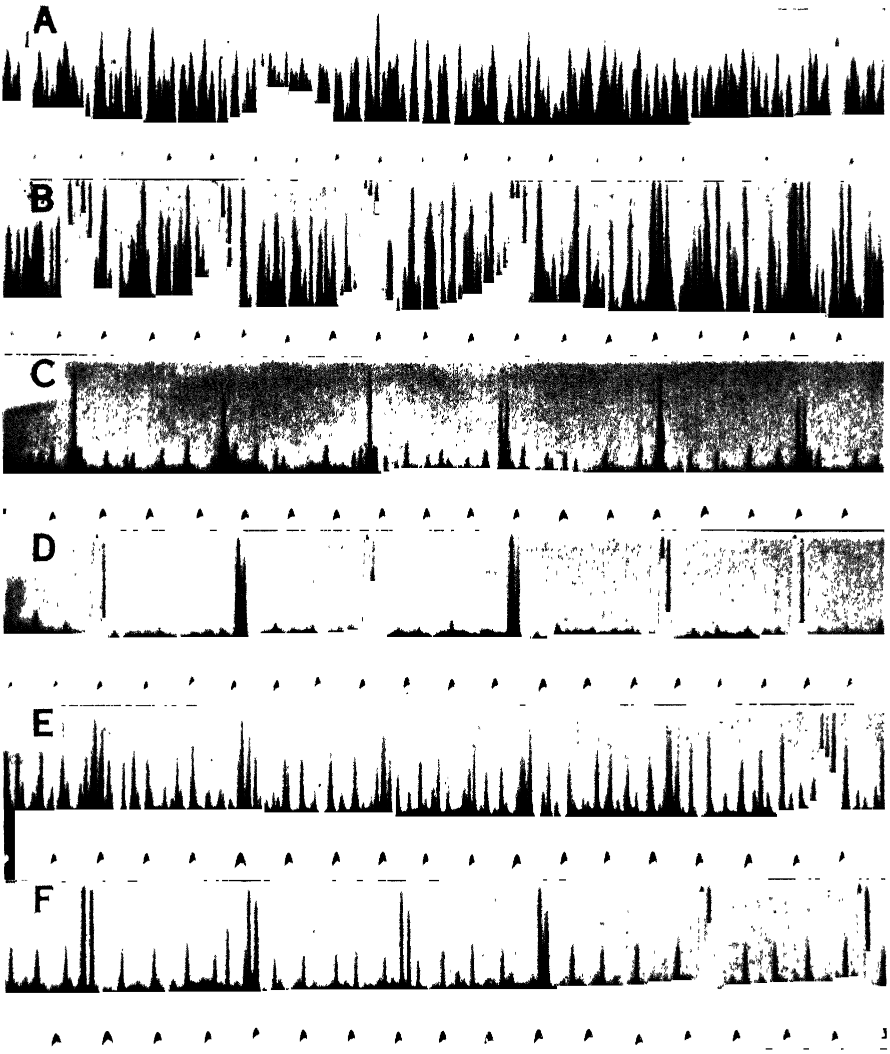

FIG. 1

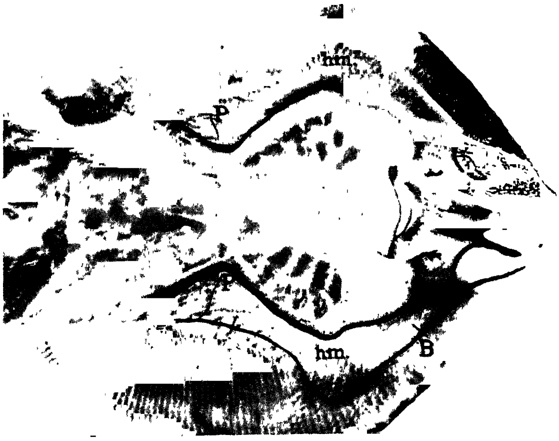

FIG. 2

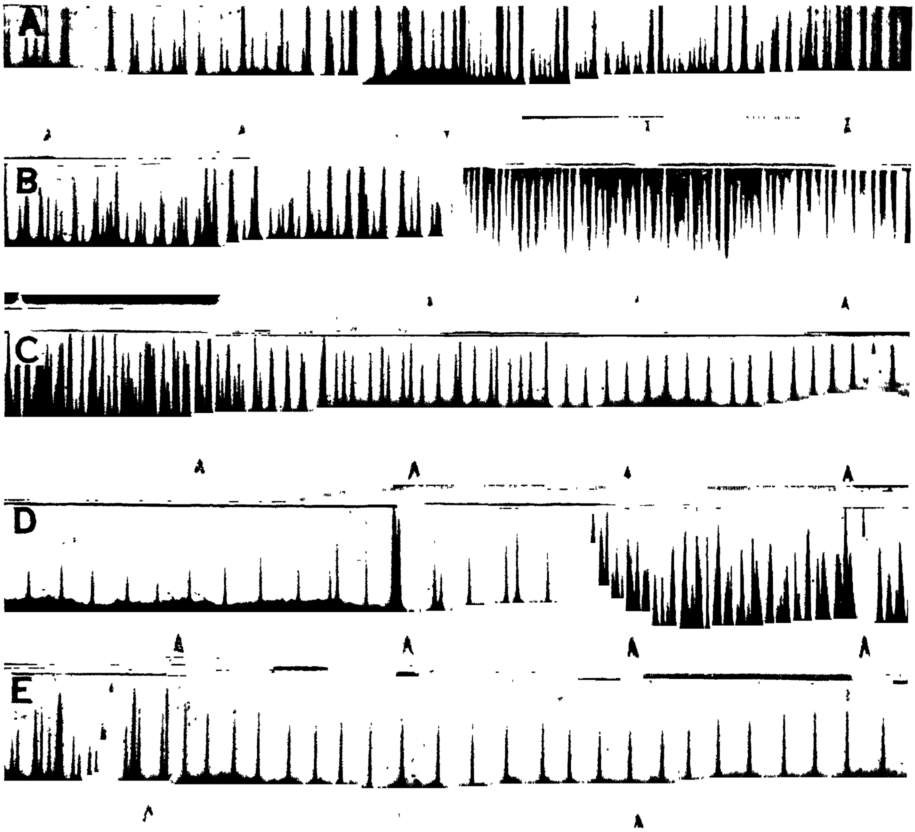

FIG. 5

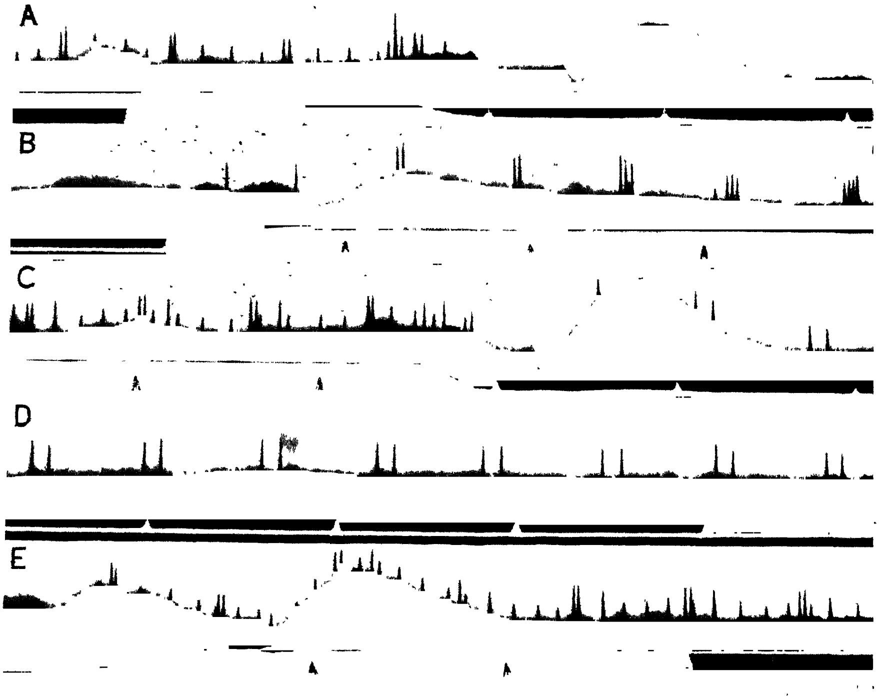

FIG. 17

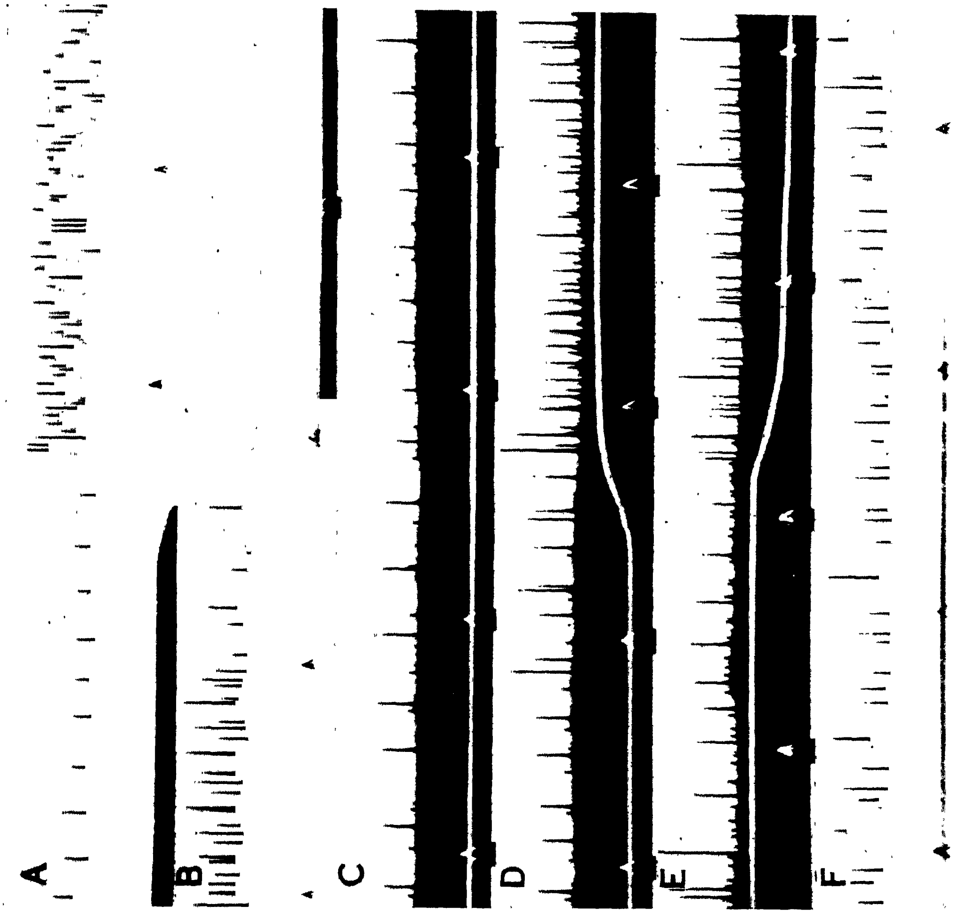

Fig. 6

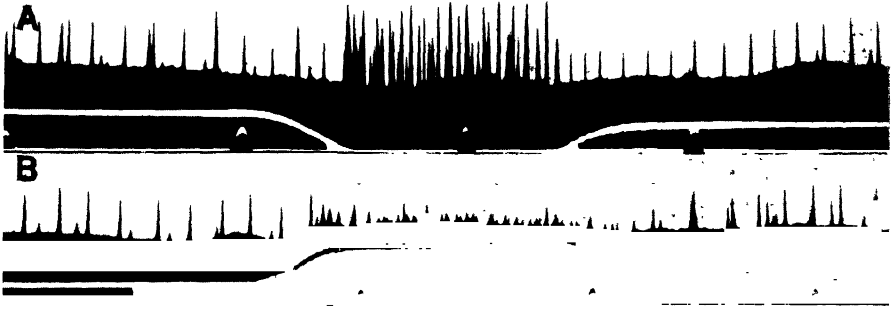

Fig. 7

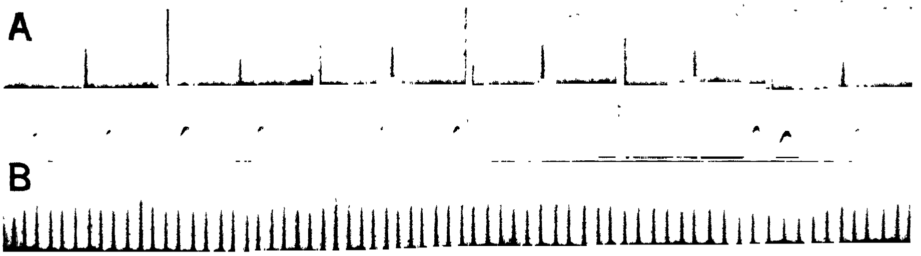

Fig. 18

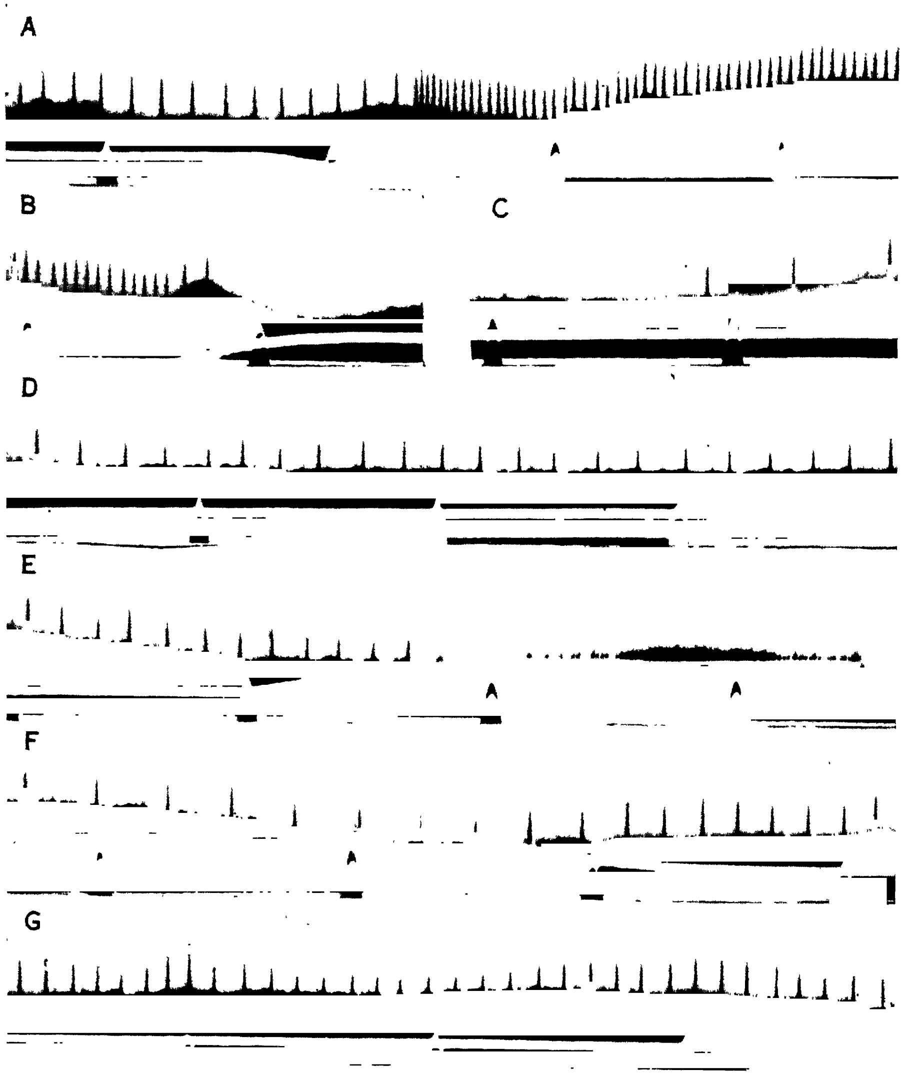

FIG. 8

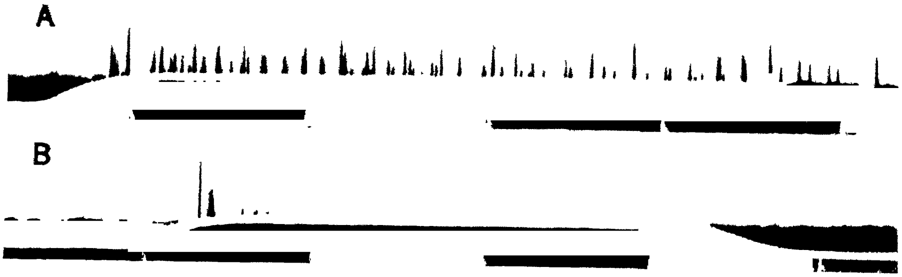

FIG. 10

is capable of serving as a touch organ, and as a detector of slow vibrations propagated through the water, and also, as Hoagland has suggested, as a proprioceptive mechanism. But fishes possess both tactile endings and muscle proprioceptors, and it is not obvious what advantage they could derive from an apparatus that is accessory to both of these. On the other hand, if vibration be the reflexly significant stimulus, it will need to be explained how the excitation arising from it is sorted out from the vigorous and constantly fluctuating afferent discharges flowing to the central nervous system along the lateral-line nerves.

Part of the apparatus was provided by a grant from the Government Grant Committee of the Royal Society.

SUMMARY

A technique has been devised for perfusing the hyomandibular canal of the ray at a constant rate of flow.

The effective stimulus for the lateral sense organs is a slow movement of the fluid in the canal.

A constant flow in the canal evokes a response which appears as an alteration in the spontaneous frequency of discharge of a single end-organ.

The response to such constant stimulation shows very slow adaptation.

The two directions of flow in a lateral-line canal are antagonistic stimuli. Flow in one direction is excitatory, flow in the opposite direction is inhibitory.

There is evidence that receptors whose behaviour with respect to the two directions of flow is antagonistic are mixed in a random assortment in the groups of sense cells in the lateral-line canals.

The possibility of arresting or reducing the spontaneous frequency of discharge by a flow of fluid in the appropriate direction implies that the inhibitory process can occur in a peripheral sensory system.

Over the greater part of the effective range of intensity of stimulation the lateral-line receptors obey the logarithmic law.

There is no evidence that any rapidly adapting receptors exist in the lateral-line canals.

The lateral-line system is exceedingly sensitive to low-frequency vibrations.

Responses to vibration are modified or completely suppressed during an inhibitory perfusion of the canal.

Direct mechanical stimulation of the lateral line as well as vibrations propagated from a distance are effective in exciting the receptors on account of the movements they cause to occur in the endolymph of the canals.

Touch and pressure applied to the skin cannot furnish a "constant" stimulation of the receptors, for the movements of endolymph which result from them are always transient.

The lateral-line system is related to the labyrinth not only in structure and development, but also in its mode of behaviour in functional activity.

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DESCRIPTION OF PLATES

Plate 19

FIG. 1—Oscillographic records showing the decline of the spontaneous activity with time in an isolated preparation of the hyomandibular canal of the ray. In all except record A the hammer was tapping at intervals of about 0·6 sec. Time signal 0·2 sec. A, spontaneous discharge 1 hr. after killing the ray. B, the same with hammer tapping. C, 2 hr. after killing. D, 3 hr. after killing. E, 3 hr. after killing, perfusion of canal and hammer tapping. F, 3 hr. after killing, spontaneous discharge after perfusion.

FIG. 2—The ventral canals of the ray injected with indian ink. Some blood spaces were accidentally injected on the right side of the snout. *hm*, loop of the hyomandibular canal. *p*, pore.

Plate 20

FIG. 5—Records showing the effect of perfusing the hyomandibular loop on the activity of a number of receptors. The white line is the perfusion signal. Its upward displacement signals tailward perfusion, its downward displacement signals headward perfusion. Time signal in seconds. Perfusion at 10 cm. water pressure. Further explanation in text.

FIG. 17—Effect of perfusion on the response to hammer taps. The hammer was tapping continuously at a rate of one tap every 0·6 sec. A, beginning of 10 sec. of tailward perfusion at a rate of 50 mm./sec. B, end of this perfusion. C, beginning of 12 sec. of tailward perfusion at a rate of 17·5 mm./sec. D, discharge during this perfusion. E, end of this perfusion. Time signal in seconds. In this figure tailward perfusion is signalled by a downward displacement of the white line. The slow undulations of the base line have no physiological significance. They were due to my having omitted to wear a rubber glove when manipulating the tap of the perfusion apparatus.

Plate 21

FIG. 6—Perfusion of preparation after reduction of the number of active units. 4½ hr. after killing. Perfusion at 10 cm. water pressure. Perfusion and time signals as in fig. 5. The records show the activity of the preparation at intervals of 1 min. when a headward perfusion of 60 sec. duration was followed by a tailward perfusion of 60 sec. duration with a rest interval of 2 min. between the perfusions.

FIG. 7—Explanation in text.

FIG. 18—Records from a single end-organ of the scapular canal of the ray. A, responses to six consecutive hammer taps. B, response during perfusion. Time signal, 0·2 sec.

Plate 22

FIG. 8—The responses of a single end-organ of the hyomandibular canal to perfusions at 6 cm. water pressure. Signals as before. A, beginning of 10 sec. headward perfusion. B, end of this perfusion. C, return of spontaneous rhythm after a silent period of 28 sec. D, the spontaneous rhythm 60 sec. after the end of perfusion in B. E, beginning of 10 sec. tailward perfusion. F, end of this perfusion. G, 10 sec. later, spontaneous rhythm.

FIG. 10—The effect of clamping the outflow tube of the perfusion circuit.

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The Sheath Components of the Giant Nerve Fibres of the Squid.

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(*Communicated by E. S. Goodrich, F.R.S.—Received 27 April* 1937)

[Plate 23]

Introduction

Recent analysis of the ultrastructure of the sheaths of vertebrate nerve fibres has revealed that from the largest, heavily myelinated to the smallest, so-called unmyelinated fibres the molecular architecture is essentially similar, the differences in optical and chemical properties being referable to the relative proportion of oriented lipoid and protein molecules (Schmitt and Bear 1937). Moreover, the birefringence varies in a continuous manner from the largest to the smallest fibres. This essential continuity of ultrastructure of the sheath reveals the distinction between myelinated and unmyelinated fibres to be entirely arbitrary.

The analysis has been extended to the sheaths of crustacean nerves, and it has been demonstrated that the metatropic† reaction of Göthlin (1913) is not, as he supposed, an artifact due to dehydration tensions, but is evidence that the ultrastructure of these sheaths likewise conforms with that of vertebrate fibres, the amount of oriented lipoid being so small that special methods are required for its detection (Bear and Schmitt 1937; Chinn and Schmitt 1937).

To extend the investigation of the ultrastructure of the sheath to a phylum other than those previously investigated, a study was made of the giant fibres recently described by Young (1936*a*) in the stellar nerves of the squid, *Loligo pealii* Leseuer. These axons are of interest in this connexion because of their enormous size, because of their unusual origin, arising as they do from the fusion of the processes of many small neurons, and because they afford an excellent opportunity for a study of single physiologically active units. Not only was it possible to demonstrate in these axons the presence of a metatropic sheath, but also finer particulars of the structure and relationship of this sheath could be observed.*

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† The term metatropic (μετατροπος, to turn around, i.e. to reverse) was introduced by Göthlin to designate fibres in which the birefringence, though positive in life, could be reversed to negative by the application of glycerine. Upon localization of this reaction to a definite sheath or layer (Bear and Schmitt 1937), this sheath has been called the metatropic sheath.

The advantages of the use of the polarization optics as an adjunct to ordinary microscopic methods are strikingly illustrated in these experiments. By comparison of fixed and stained sections with the appearance in polarized light of the same structures in the fresh axon, details of structure and of ultrastructure have been observed which would probably have escaped detection by the use of either method alone.

Methods

Radiating from each stellate ganglion are about ten giant fibres supplying the musculature of the mantle (Young 1936*b*). For these experiments the giant fibre in the nerve passing to the hindermost portion of the mantle has been used, since it is the largest and may be dissected for considerable lengths with relatively little damage. From squids of average size one readily obtains axons 0·4–0·6 mm. in diameter and 60–70 mm. long. With fine scissors the surrounding muscle, connective tissue and most of the smaller nerve fibres can be removed, leaving the giant fibre which is then ligated at both ends and cut beyond the ties; this ligation being essential because of the ease with which the axoplasm flows from cuts. After removal from the body the fibre is placed on a slide in sea water and teased as free as possible of all remaining smaller fibres, blood vessels and connective tissue under the binocular dissecting microscope. The fibres are so large that for many purposes they can be studied with low-power objectives (6 × and 20 ×), after immersion in the appropriate solutions on strain-free slides. To reveal the structure of the sheaths use is made of a compression instrument consisting essentially of a piece of microscope slide and a cover-slip, fastened to metal supports which are hinged together in such a manner that by turning a screw the distance between the glass surfaces can be varied and the fibre thus flattened to any desired extent. Under these conditions observations with powers up to oil immersion are possible.

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* To avoid confusion in the terms used to describe the parts of a nerve fibre we have adhered to the following terminology. "Axon" refers to the process of the neuron and is synonymous with "axis cylinder" (see *Oxford English Dictionary*). The term axon, therefore, does not include any enveloping elements except the problematical axiolemma. The substance of the axon will be called "axoplasm", by analogy with protoplasm, meaning the whole substance of the axon, without reference to any hypothetical substance supposed by some to surround the neurofibrils.

A standard Leitz model GM polarizing microscope was used. For ordinary observations of the sign of birefringence the gypsum Red I plate is suitable, while in cases where the retardations are quite small a rotating mica plate compensator with a retardation of $\lambda/20$ has been found convenient. Measurements of fibre dimensions were made with a Leitz filar micrometer.

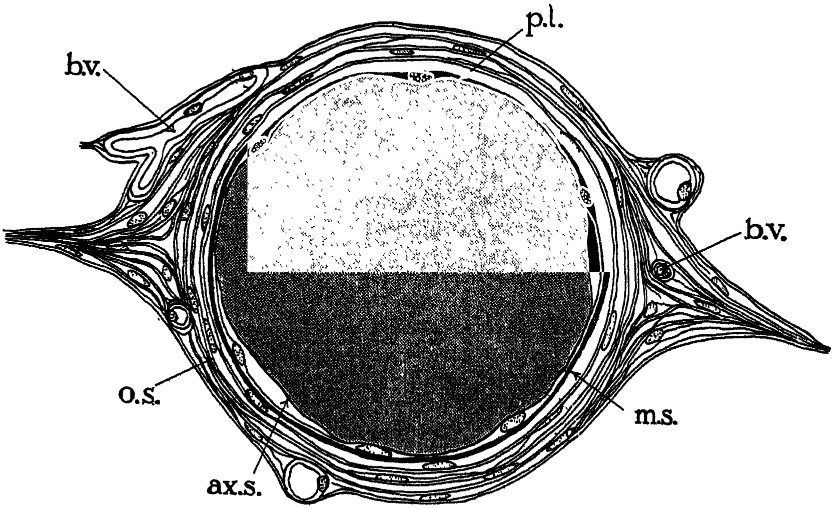

FIG. 1—Diagram of transverse section of a giant fibre from a stellar nerve of *Loligo pealii*. The metatropic sheath, *m.s.*, is shown in black, but in histological preparations it is indistinguishable from the outer layer of collagenous tissue, *o.s.* The surface of the axis cylinder, *ax.s.*, in a number of places is shown as if shrunken away from the sheath, as it is seen in many preparations, though in life it completely fills the tube. The cells of the protoplasmic layer, *p.l.*, are shown as a syncytium, as they appear in sections, though it is difficult to be certain that fine boundaries do not occur between them. Several blood vessels, *b.v.*, are also indicated.

EXPERIMENTAL RESULTS

The investments around the giant axons in the squid may be considered under three general categories, beginning from the outside (see fig. 1): (1) Endoneural tissue comprising several wrappings of fibrous connective tissue and containing blood vessels. (2) A sheath containing oriented lipoid and protein, presumably analogous to the myelin sheath of vertebrate fibres. This smooth, myelin-containing component is the structure to which we apply the term "metatropic sheath". (3) An internal protoplasmic layer, probably comparable to the Schwann cells of vertebrate fibres, except that it lies inside rather than outside the myelin-containing sheath.

1—*Connective Tissue*

The relative amount of closely adhering endoneural tissue, which may be seen best in cross-sections of fixed preparations (fig. 2, Plate 23), is never as extensive as in the case of certain crustacean fibres (Young 1936*c*; Bear and Schmitt 1937). The sheets are wrapped about the giant fibre in layers containing strands extending preponderantly either in longitudinal or circular directions. While the outer strands, which form a loose network, can readily be teased off, the closely adhering inner layers can be removed only with great difficulty. By taking advantage of the collagenous nature of these layers and immersing the fibre in acid or alkali, the connective tissue may be made to swell to such an extent that teasing is greatly facilitated. In 0·01N NaOH, for example, as the outermost layer swells it can be teased away; this then permits the next layer to swell, after which it can in turn be removed, and so on. It has been found impossible, however, to remove all of the connective tissue down to the metatropic sheath without puncturing the axon. The innermost layers, consisting mainly of strands wound in circular or spiral directions, adhere to the giant nerve fibre with great tenacity.

The general course of the connective tissue strands may be clearly demonstrated by the use of polarized light. Each collagen strand is uniaxially positive with respect to its long axis. Viewed laterally the fresh giant fibre manifests positively birefringent borders, due primarily to the longitudinally disposed connective tissue (and in part to the metatropic sheath). That which is circularly wound is optically ineffective at the edges of the giant fibre, for in this position the optic axes of the strands are parallel to the axis of the microscope. In the central region of the giant fibre the circularly disposed connective tissue gives rise to negative birefringence, since, although individual filaments are positive, they are oriented perpendicularly to the long axis of the axon and therefore make the latter appear to be negative with respect to its length (fig. 3, Plate 23). In the fresh condition this weak negativity contributed by the connective tissue strands is overbalanced by the positivity of the axis cylinder itself, but under any condition in which the organization of the axoplasm is destroyed, such as application of alkali, long standing in water or sea water, etc., the central negativity caused by the sheath appears. If the fibre be slit longitudinally and its wrappings laid out like a sheet, the course of the connective tissue strands may be clearly seen by the use of the rotating mica plate compensator. Compensation to darkness of the filaments running in one direction throws into sharp contrast those running in the perpendicular direction, which, under these conditions appear bright (fig. 4, Plate 23). The circularly disposed strands make up by far the stronger portion of the wrappings. This can be shown by tearing experiments. If needles are inserted in two ends of a giant fibre and the latter is then subjected to longitudinal tension, breaking always occurs at right angles to the length. On the other hand, if the two needles are placed near together and the tension applied at right angles to the length, they cut through the investments to produce a break which again is perpendicular to the fibre axis.

Changes in solvation of the connective tissue, particularly the circularly wound filaments, may result in sphincter-like constrictions of the giant fibre. Such constrictions often occur as a result of injury during preparation. They also tend to limit the egress of axoplasm from small cuts by closing up the wound. Owing to the circularly and longitudinally oriented wrappings of connective tissue the giant fibre as a whole shows little thermal shortening or swelling shortening in thiocyanate, the axis cylinder being unable to cause the whole structure to contract. Small pieces of sheath isolated from the axon show considerable shortening.

2—*The Metatropic Sheath*

The existence of a myelin-containing sheath may be demonstrated in a number of ways. Since it does not swell appreciably in alkali, as does the surrounding connective tissue, considerable contrast between the structures may be produced by the use of NaOH. In a molar solution the birefringence of the metatropic sheath changes from positive to negative with respect to the long axis, owing to the relatively high refractive index of the alkali and possibly to a partial degradation of the metatropic sheath protein. Since the swollen connective tissue strands retain a weak positivity under these conditions, examination between crossed Nicol prisms with the gypsum plate or rotating mica plate compensator heightens the contrast.

The metatropic sheath is also brought out by immersing the fibre in mixtures of glycerine and sea water. Because of the relatively high refractive index of the glycerine solution, the form birefringence of the non-lipoid portion of the sheath disappears and the micellar birefringence of the lipoids becomes manifest by the reversal of the sign of birefringence from positive to negative (fig. 5, Plate 23). This micellar birefringence is due presumably to the presence of lipoid micelles oriented with optic axes radial. When glycerine solutions are thus applied, considerable shrinkage takes place with the result that the metatropic sheath is thrown into longitudinal folds and shows a rough or layered appearance with no sharply defined inner boundary. To estimate the thickness of the sheath we have found it convenient to compress the fibre somewhat in the instrument especially designed for this purpose. Pressure is applied until the wrinkles in the sheath become unfolded by pressure from the axoplasm, at which time the sheath appears as a thin homogeneous layer with clear-cut boundaries. Under these conditions the thickness of the metatropic sheath has been estimated to be something less than 1 % of the fibre diameter, being about 3–4μ thick in axons with 400–500μ diameters (fig. 6, Plate 23). That the tensions set up by the compression instrument are not responsible for the negativity is shown by the fact that even considerably greater tensions exerted on axons treated with subliminal concentrations of glycerine showed no reversal.

It has been pointed out (Bear and Schmitt 1937) that information as to the mechanism of the metatropic reaction and the composition of the metatropic sheath may be derived from a study of the minimal concentration of glycerine and other reagents needed to produce reversal of birefringence. Considerable difficulty was encountered in determining this minimal concentration with the giant nerve fibres because of a high degree of variability from one nerve to the next. In the average, however, 35–40 % glycerine in sea water is the minimal concentration. The comparable figure for arthropod nerves (and for the smaller fibres in squid nerve) is 30 % or slightly lower. In so far as these figures are reliable they indicate the presence of relatively less lipoid in the squid giant fibre sheaths than in the corresponding structures of typical arthropod fibres.

To prove conclusively that the reversal is due to lipoids it would be desirable to show, as has already been done with lobster nerves, that preliminary treatment of the axon with alcohol or other lipoid solvents abolishes the effect. It was discovered, however, that treatment with alcohol causes the axon to shrink away from the sheath as a compact rod, as will be described in detail in another connexion. This renders difficult any observations on the birefringence of the sheath, particularly because it is no longer possible to compress the fibre properly on account of the shrinkage of the axis cylinder.

As in the case of lobster nerve, after standing in sea water the minimal concentration of glycerine required for the metatropic reaction with squid fibres may be considerably increased. This effect is doubtless due to a gradual disorientation of the lipoid micelles.

3—*Inner Protoplasmic Sheath*

In cross-sections of fixed preparations (fig. 2), both in the giant and in the smaller axons, nuclei were observed bordering on and indenting the axis cylinder (Young 1936*c*). After the discovery of the metatropic sheath it was obvious that these nuclei could not belong to the connective tissue layers investing the axon as was originally believed. In fresh preparations, moreover, especially in large axons stained with methylene blue and compressed somewhat between glass plates, it is possible to see that these nuclei belong to a very thin protoplasmic layer lying between the axis cylinder and the metatropic sheath; the latter has a smooth contour, without irregularities, and has never been observed to contain nuclei within its thickness. It appears, therefore, that the inner protoplasmic layer with its nuclei corresponds to the Schwann sheath of medullated nerve in its close relation to the myelin-containing layer, except that it lies inside rather than outside the latter.

Discussion

The demonstration of the presence of a metatropic sheath in the squid giant nerve fibre adds evidence from an additional phylum of the widespread occurrence in invertebrate nerve of lipoid-containing structures similar in composition and ultrastructure to the myelin sheath of vertebrate fibres. The value of the polarizing microscope in enabling the detection of this structure where ordinary histological methods have failed, doubtless, owing to the small amount of lipoid present, is strikingly illustrated.

The presence of this sheath about the squid giant axons is significant, moreover, as evidence for the unitary nature of this fibre. In all known cases of nerve fibres with metatropic or myelotropic sheaths the lipoid-containing structure surrounds a single conducting unit, hence from the optical experiments it appears that, despite their origin from the fusion of the processes of many small neurons within the stellate ganglia, the giant axons of the squid mantle nerves are individual physiological units. In fact, after the discovery of the metatropic sheath of these axons, it was found that this is the case, the axons responding to electrical stimulation in all-or-none manner (Young, 1937).

The presence of the protoplasmic layer connected with the metatropic sheath is another point of similarity between the squid axons and other widely different types of nerve. Unlike the Schwann sheath of vertebrate fibres, however, the cells are located inside instead of outside the associated myelin-containing region. A similar inversion has been described by Retzius (1890) for the nerves of prawns and shrimps, which have heavily myelinated fibres (without nodes of Ranvier).

Whether or not this inverted position of the protoplasmic layer is typical of invertebrate nerve fibres in general it is too early to say. Nevertheless, the inverted position in these several cases would point to the necessity of considering, in theories regarding the electrical properties of nerve, the possibility of interposition of an additional layer of protoplasm between the axon proper and the "insulating" lipoid-containing sheath.

SUMMARY

Examination of fresh giant nerve fibres of the squid, *Loligo pealii*, by means of polarized light reveals, in addition to the collagenous investment, a myelin-containing layer similar in optical properties to the metatropic sheaths previously demonstrated in crustacean fibres. This layer is analogous to the myelin sheath of vertebrate fibres with respect to the orientation of lipoid and protein molecules, the chief differences being the relative thinness and low lipoid content of the sheath in *Loligo*.

The differentiation of this layer from those of the surrounding connective tissue permitted identification of nuclei, lying immediately next to the axis cylinder, as belonging to cells presumably analogous to the Schwann sheath cells of vertebrate fibres. As in the fibres of prawns and shrimps and as opposed to the relation in vertebrate axons, these cells lie inside the myelin-containing layer, being placed between it and the axis cylinder. This fact must be borne in mind in any attempt at a localization of "the irritable membrane" in axons generally.

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DESCRIPTION OF PLATE 23

FIG. 2—Transverse section of the hindermost stellar nerve of *Loligo pealii* in the region of the giant fibre. The preparation was fixed in Flemming's fluid and stained with Mallory's stain. Compare with fig. 1 in text. The region in which the metatropic reaction occurs is shown at *m*; notice, however, that it cannot be distinguished in such preparations from the outer sheaths, *o.s.* Near the lower left corner of the giant fibre is seen one of the nuclei (*n*) of the protoplasmic sheath indenting the axoplasm (*ax*). Magnification 285 ×.

FIG. 3—Deteriorating isolated giant fibre in sea water photographed in polarized light. The crossed Nicol prisms are oriented as indicated by the cross-hairs, and the rotating $\lambda/20$ plate compensator has been set so that the negative regions of the fibre, where the axis-cylinder deterioration is complete (ax_1), are compensated to black. The bright central portion (ax_2) is characteristic of the positive fresh axon. Magnification 25 ×.

FIG. 4—A portion of the investments removed from the axis cylinder of an isolated axon which had been fixed in 10 % formalin. The sheath has been pressed flat between slide and cover-slip, and photographed in polarized light, the compensator having been set to blacken the positive (vertical) connective tissue fibres which extend in the direction of axon length. Most of these have been removed during preparation, however, leaving preponderantly the smaller circularly wound fibres, which in the figure appear as bright, horizontal striations. Magnification 90 ×.

FIG. 5—Edge of a giant fibre after treatment with glycerine. The fibre has been subjected to moderate compression between glass plates and photographed in polarized light. The compression in this case is insufficient to unfold the wrinkled sheath completely, but the negative metatropic layer can be seen as a rough black line (*m*) on the inner side of the outer sheath (*o.s.*). Between the black metatropic sheath and the bright positive region of the axis cylinder (*ax*) (on the left) is a narrow space (*f*) wherein fluid pressed out of the axis cylinder was seen to flow as the compression was changed. Magnification 85 ×.

FIG. 6—A preparation similar to that of fig. 5, except that the axon has been better cleaned of connective tissue and subjected to much greater pressure. The separation between the glass plates is now so reduced that only the negative metatropic sheath (*m*) appears definitely birefringent, its black appearance under the optical conditions employed indicating negative character. Folds in the sheath have been pressed out and the appearance is a better indication of the true thickness of this sheath. Magnification 180 ×.

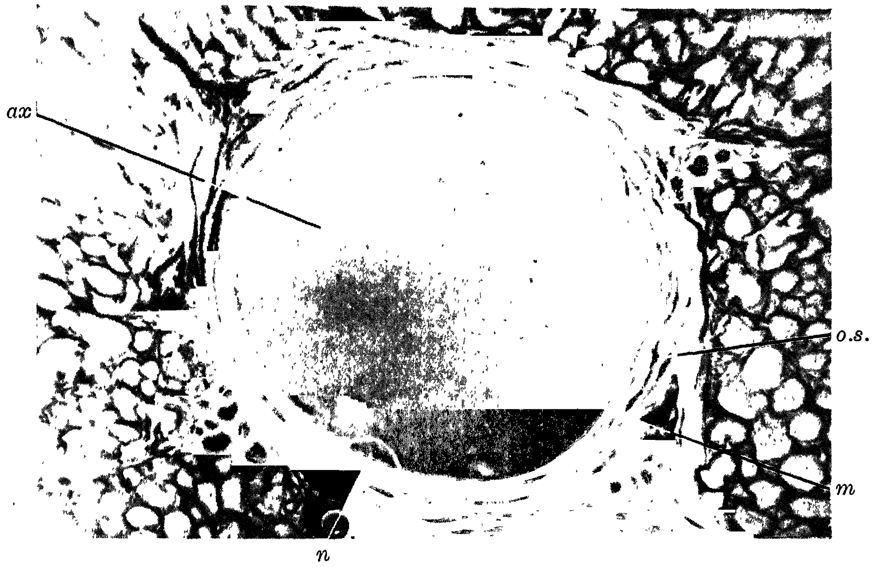

FIG. 2

FIG. 4

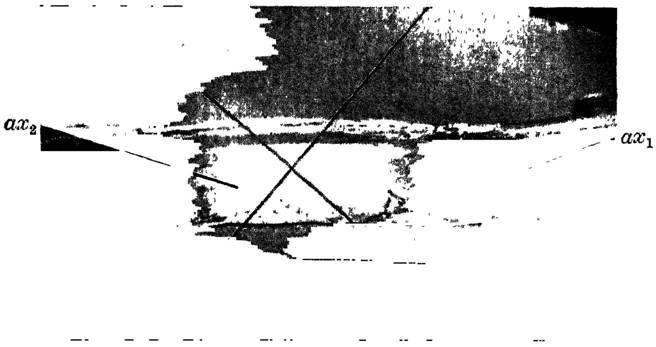

FIG. 3

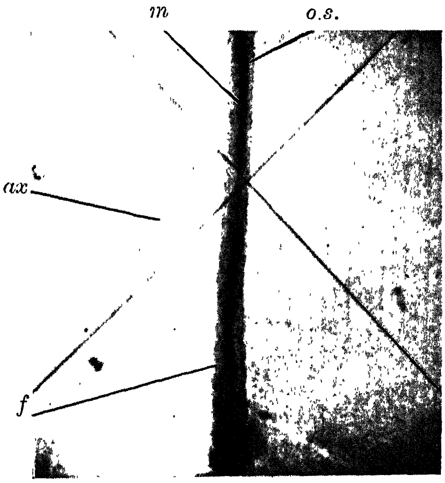

FIG. 5

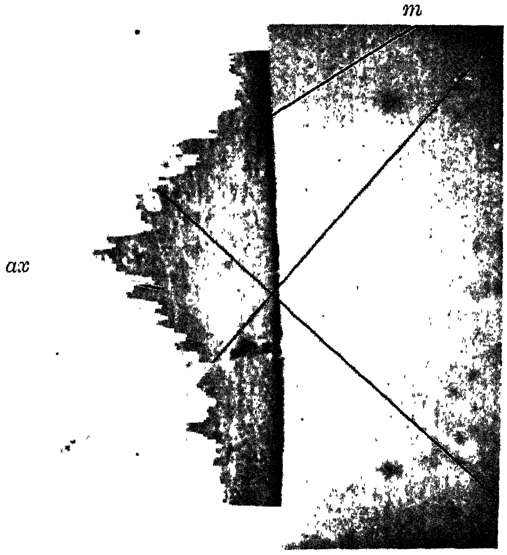

FIG. 6

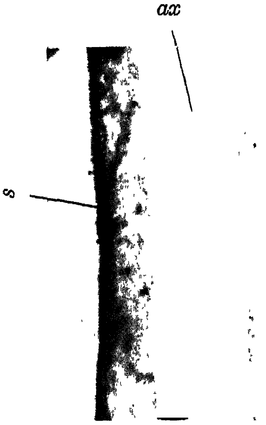

FIG. 3

FIG. 5

FIG. 7

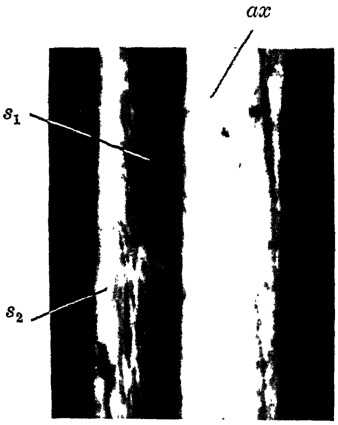

FIG. 4

FIG. 6

FIG. 8

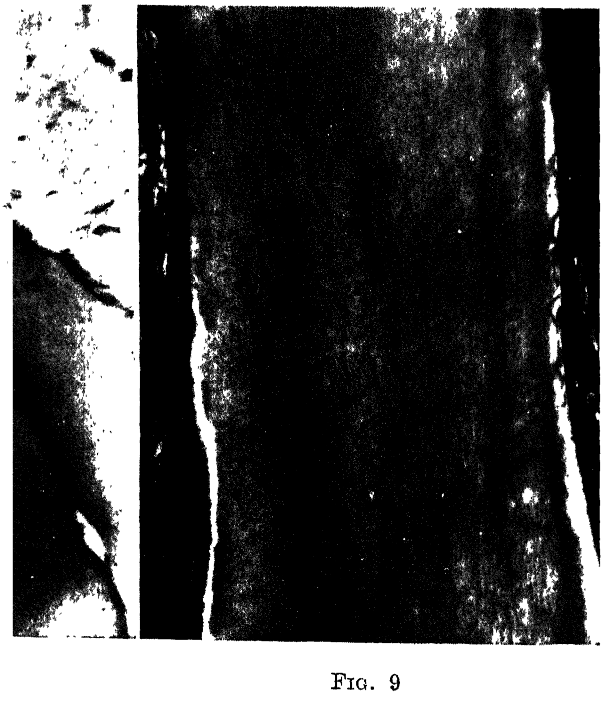

FIG. 9

611.018.83

The Ultrastructure of Nerve Axoplasm

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(*Communicated by E. S. Goodrich, F.R.S.—Received* 27 *April* 1937)

[Plate 24]

Introduction

In previous papers on the micellar organization of the nerve fibre, attention has been directed almost entirely to the lipoid-containing sheaths. Polarized light and X-ray diffraction studies have emphasized the similarity of the architecture of these structures over a wide range of animal forms, from the highly myelinated nerves of vertebrates to the so-called non-medullated fibres of invertebrates (Schmitt, Bear and Clark 1935; see preceding paper for other references).

To date little more than a beginning has been made with analysis of the ultrastructure of the axoplasm. This has been due largely to technical difficulties arising from the small size and weak birefringence of the axons so far studied, from the unfavourable position of the axis cylinder within more or less complex sheaths, and from the extreme sensitivity of this colloid to the action of reagents. Valentin (1861), Klebs (1865), v. Ebner (1882), and Friedlaender (1889), found no birefringence in the fresh axis cylinder, although Valentin mentioned that coagulation produced slight positivity. Ambronn (1890) described a positive birefringence common to all types of nerves and refers this to a "ground substance". Though Apathy (1890) in early works concluded that neurofibrils are optically isotropic, in later studies (1897) he considered that the fibrils were uniaxially positive with respect to their length and demonstrated dichroism of the neurofibrils in gold preparations. Finally, Göthlin (1913) showed beyond doubt that the axis cylinder is weakly uniaxially positive in life, particularly at the regions of nodal constriction (vertebrate axons).

The only unequivocal conclusion that emerges from these investigations on axis cylinder birefringence is that in life this structure contains a certain amount of anisotropic organization, disposed symmetrically with respect to the long axis of the fibre. Although as yet no quantitative data have been recorded on the birefringence of the fresh axoplasm, its relative weakness is obvious from the failure of the early workers even to detect it. This fact, together with the failure to date of X-ray diffraction studies to yield evidence for any considerable degree of orientation in the living axis cylinder (Boehm 1933; Schmitt, Bear and Clark 1935), does not fit in well with the histological data showing copious well oriented fibrillation easily produced under abnormal conditions. Since many have been led to regard the living axon as a colloidal system containing the fibrils in latent form (see Peterfi's review 1929), it is of considerable importance that the conflicting histological and optical data be reconciled.

In the course of the experiments reported in the preceding paper it became clear that the squid giant fibre is ideal for an attack on the problem of the organization of the living axoplasm by means of the sensitive method of polarization optics. Particularly its large size favours application of quantitative methods. While the data presented in the present paper do not lead to an unique interpretation of axis cylinder organization, they restrict the possible micellar configurations which need be considered in further investigations.

METHODS

In the preceding paper descriptions were given of the preparation of the squid giant nerve fibres as well as of the optical equipment and the instrument for application of pressure to the axons. To prepare the specimens for determination of form birefringence, single fibres, teased as free as possible from extraneous material, were examined immediately and measured with respect to diameter and retardation of the central portion as viewed laterally under the polarizing microscope. These were then placed in various fixing agents, those most suitable for the present purposes being 5 % $HgCl_2$, 10 % formalin (both in sea water), or 35 % ethyl alcohol. Those in $HgCl_2$ or formalin lost little of their original diameter, but those in the alcohol exhibited considerable shrinkage of the axon from the investing layers (see fig. 3, Plate 24). The alcohol-fixed axons could then be separated from their investments (fig. 5, Plate 24), with no damage except at the ends where manipulation produced injuries, while the $HgCl_2$- and formalin-fixed axons could not be so removed. The latter samples and the isolated fixed axons were then run up slowly to absolute alcohol, after which they were ready for immersion in fluids of various refractive indices.

Some of the sheath-free axons were also further shrunk by exposure to air for a few seconds before immersion (fig. 6, Plate 24).

The observations, consisting of the determination of retardation and diameter at various positions along each axon, were averaged to obtain the final figures taken as typical of the preparation. Each sample, a portion several millimetres long, was transferred from one immersion medium to another, instead of placing similar portions in separate media. In addition, each was investigated by immersion first in media of increasing refractive indices followed by return in the direction of decreasing indices. In the case of the $HgCl_2$- and formalin-fixed fibres it was necessary to correct for a certain amount of birefringence due to connective tissue (cf. fig. 4, Plate 24). This was done by immersion of sheaths of alcohol-fixed fibres from which the axon itself had been removed, the observed retardations (with rotating $\lambda/20$ plate) then being subtracted from those obtained with the complete fibres. Immersion for 2 hr. sufficed to yield constant measurements, though usually a period several times longer was allowed. Refractive indices of the immersion fluids and mixtures used (see Table I) were determined directly from samples of the liquids by means of an Abbé refractometer or, in the cases of excessively high index, by microscopic determination of the apparent decrease in distance separating two parallel glass surfaces when the liquid was substituted for air between them. Retardations were measured by the Sénarmont $\lambda/4$ plate method. White light was used, and in the calculations of retardations the wave-length 551mμ was employed.

EXPERIMENTAL RESULTS

Birefringence of Fresh Axoplasm

In the freshest condition the entire giant fibre possesses positive birefringence with respect to its length, as viewed laterally. Upon standing in sea water the birefringence of the central region, looking through the axis cylinder, decreases and eventually reverses sign, becoming slightly negative. Fig. 1 shows the time course of this deterioration in a typical experiment. As shown in the previous paper, the negativity is a property of the connective tissue rather than of the axon, hence in order to obtain the birefringence changes of the latter alone it is necessary to correct for the relatively constant connective tissue contribution (see dotted line, fig. 1). Thus, in the case of the example given, the retardation attributable to the fresh axoplasm alone is 65mμ, after adding a sheath correction of 7mμ. Upon dividing this retardation by the diameter of the axon (445μ), one obtains a birefringence of about $1 \cdot 5 \times 10^{-4}$, which is also the average obtained from similar measurements on twelve other fibres. The birefringence of the axoplasm is, therefore, seen to be rather low, being 3% of that of the *Q* band of muscle, 5×10^{-3} (Fischer 1936).

There can be no doubt of the pre-existence of this birefringence in a normal axon. Measurements have been made on fibres still attached to the mantle muscles and in which excitability was demonstrated by the vigorous contraction of the muscles in response to stimulation of the nerve by weak induction shocks applied proximal to the region being observed microscopically. The positivity was of the same order as that given above for extirpated, fully dissected and teased fibres.

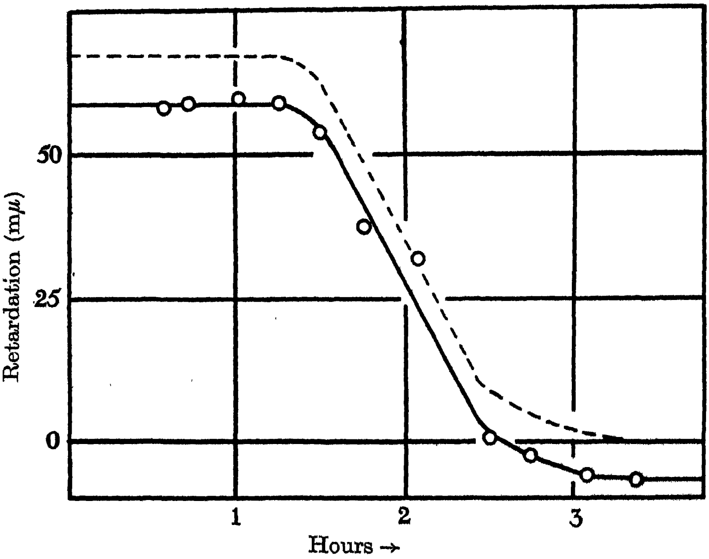

Fig. 1—Curve showing changes of axon retardation with time (in sea water). The solid line with circles represents the experimental observations; the dotted line shows the axis cylinder changes obtained by correcting for the relatively constant connective-tissue factor.

Birefringence in Relation to Fibril Formation

When birefringence is retained after fibril formation, it appears to be localized definitely in the fibrils. This is particularly strikingly illustrated in the case of the abundant fibrillation which may be produced by compression of fibres between glass plates. The fibrils are arranged in bundles with occasional waves and whorls in their course, and it is likely that their formation is to be ascribed, in part at least, to the flow of axoplasm (see

Young 1936*c*) produced during compression. At any rate, after having been formed, the bundles can be seen to move about as in units during slow compression and decompression, the fibril groups retaining their identity whilst uncoiling and coiling. The fibrils possess positive birefringence with respect to their length, and show a negative cross wherever coiling occurs.

It does not follow, however, that when fibril formation is pronounced, birefringence also will be striking. The best example of this fact comes from experiments in which fibres are subjected to the action of half-saturated urea solution. Immediately the axon begins to shrink, receding from the sheath until its diameter is about half the initial value. At this point the birefringence is quite marked and still positive. Within a few minutes, however, the shrunken axon begins to expand, as the urea penetrates, until it occupies once more the original volume. During the expansion the birefringence fades away completely and permanently, despite the presence of profuse fibrillation; transferring such a preparation to a medium of low refractive index, such as water, brings out the fibrils but does not restore the birefringence.

Another case of fibril formation with accompanying loss of birefringence is that observed when fibres are subjected to isotonic salt solutions buffered to weakly alkaline reaction. Though disintegration of the colloids of the axoplasm is eventually complete, at a certain point in the series of events (about 45 min. after initiation) it often happens that birefringence will have disappeared even though fibrils are still abundant.

Both types of fibril formation without retention of birefringence are brought about by reagents that are well known to produce changes in protein structure. This suggests that the transition from anisotropic to isotropic fibrils results from alteration of molecular linkages that are important in maintaining the structure of the protein micelles to which the original birefringence was due. Muralt and Edsall (1930) observed a similar destruction of the anisotropy of myosin micelles by urea and alkali. On the other hand, good fixatives, such as alcohol, formalin, $HgCl_2$, etc., preserve the essential organization, the changes which they produce having little effect on the birefringent properties of the micellar structure. In muscle (Noll and Weber 1934) fixation does not greatly alter the birefringence quantitatively, and below it is shown that this is true also of fixed nerve axons if the effects of shrinkage are considered.

Analysis of Axoplasm Birefringence

In order to determine the cause of the birefringence of axoplasm, the technique of immersion in fluids of different refractive indices must be

TABLE I—IMMERSION DATA FOR AXIS CYLINDERS OF SQUID GIANT NERVE FIBRES

	Refractive index of immersion fluid	Axon fixed in $HgCl_2$				Axon fixed in alcohol						Axon fixed in alcohol and dried in air		
Immersion fluid	n_2	Diam. μ	Ret. mμ	Cont. of sheath mμ	Biref. × 10^5	Diam. μ	Ret. mμ	Biref. × 10^5	Diam. μ	Ret. mμ	Biref. × 10^5	Diam. μ	Ret. mμ	Biref. × 10^5
CH_3OH	1·329	384	95	11	24·7+	232	154	66·5+	257	128	49·9+	128	84	65·6+
C_2H_5OH	1·362	397	73	10	18·4+	227	120	53·0+	—	—	—	—	—	—
C_3H_7OH	1·385	368	62	9	16·8−	217	101	46·6+	250	90	36·0+	122	69	56·5
$CHCl_3$	1·443	382	36	7	9·7+	219	71	32·4+	258	67	25·9+	120	57	47·5−
$C_6H_4(CH_3)_2$	1·495	379	18	4	4·8	214	53	24·7	239	49	20·5−	118	51	43·2−
C_6H_6	1·501	391	16	4	4·1+	208	40	19·2−	—	—	—	—	—	—
$(CH_2Br)_2$	1·537	382	10	4	2·6+	210	37	17·6−	—	—	—	—	—	—
$\frac{3}{4}$ Xyl., $\frac{1}{4}$ MI	1·546	384	13	4	3·4+	208	38	18·2−	244	35	14·3−	118	49	41·6−
$\frac{3}{4}$ EBr., $\frac{1}{4}$ MI.	1·581	379	10	4	2·6	207	32	15·4−	—	—	—	—	—	—
$\frac{1}{2}$ Xyl., $\frac{1}{2}$ MI.	1·601	379	12	5	3·2	216	32	14·8+	239	30	12·5−	122	43	35·2
$\frac{1}{2}$ EBr., $\frac{1}{2}$ MI.	1·629	367	14	5	3·8−	207	40	19·3−	—	—	—	—	—	—
$\frac{1}{4}$ Xyl., $\frac{3}{4}$ MI.	1·660	379	25	6	6·6	212	55	25·9−	243	44	18·1−	121	48	39·7−
$\frac{1}{4}$ EBr., $\frac{3}{4}$MI.	1·680	367	37	8	10·1−	206	65	31·6−	—	—	—	—	—	—
MI.	1·743	367	47	9	12·8−	212	89	42·0−	240	72	30·0−	128	70	54·6+
Av. diam. fixed preparations	—	379	—	—	—	214	—	—	246	—	—	122	—	—
Fresh axons in sea water	1·34	422	53	7	12·5	476	60	12·0	428	60	14·0	428	60	14·0

Diam. = diameter; Ret. = retardation; Biref. = birefringence.

employed. Obviously, because of the lability of the fresh axon this cannot be done on normal unfixed material. However, by comparing preparations fixed in different ways and shrunk to varying extents, it is possible to obtain information concerning the ultrastructure of the *living* axon.

In Table I are given the immersion data as obtained from typical preparations. Each value for retardation and diameter represents the mean of several (six to eighteen) readings in various localities along the axis cylinders. The $HgCl_2$-fixed preparation is typical also of formalin-fixed material in the general shape and location of the birefringence curve. The two alcohol-fixed samples serve to compare similar preparations of different axons. The alcohol-fixed strand at the right of the table was from a portion of the axon adjacent to that which furnished the strand immediately to its left, except that it had been dried in air. The column of sheath corrections in the case of the $HgCl_2$-fixed material has already been added to the retardation given. The data are plotted in fig. 2. In spite of all precautions, the results do not lie strictly on smooth curves. The chief errors are those involving the measurement of diameters, although some fluctuations may be natural effects of the immersion fluids (oriented imbedding, etc.). One rather important effect, seen best in Table I, is the tendency, which seems greater than experimental error, for the fluids of low refractive index to cause measurement of diameters larger than average. Correction for the differences in states of swelling of the axis cylinders would have the effect of raising the curves of fig. 2 in the region of low n_2. The direction of correction for this effect, which would make the data applicable to samples of constant diameter equal to the average, is indicated in the table by plus and minus signs following the birefringence values. Because of these several difficulties, the scope of the following analysis has been limited so as to be independent of the variability and errors mentioned.

It is at once evident from the curves of fig. 2 that the birefringence of the fixed axis cylinders is produced by two effects: (1) an intrinsic or "eigen" birefringence of certain micelles themselves, and (2) a form birefringence resulting from the anisotropic shapes of these micelles. The former is independent of n_2, the refractive index of the immersion medium, hence is indicated by the ordinates of the minima, while the latter does depend upon n_2, showing as a function thereof the characteristic parabola-like curve. The signs of both are positive. The minima appear in the neighbourhood of 1·57–1·60, which indicates the mean refractive index of the micelles involved, pointing to their protein-like nature (cf. Weber 1935, who found 1·576 for muscle myosin, and Chinn and Schmitt 1937, whose minima for nerve sheath proteins occurred at 1·58).

Closer examination of the curves indicates other interesting relations. In going upward from the n_2 axis the curves encountered belong to preparations which are more and more shrunk from their original fresh-axon condition. This can be seen from Table I, wherein are given the fresh axon sizes and the average diameters after preparation.* It then becomes

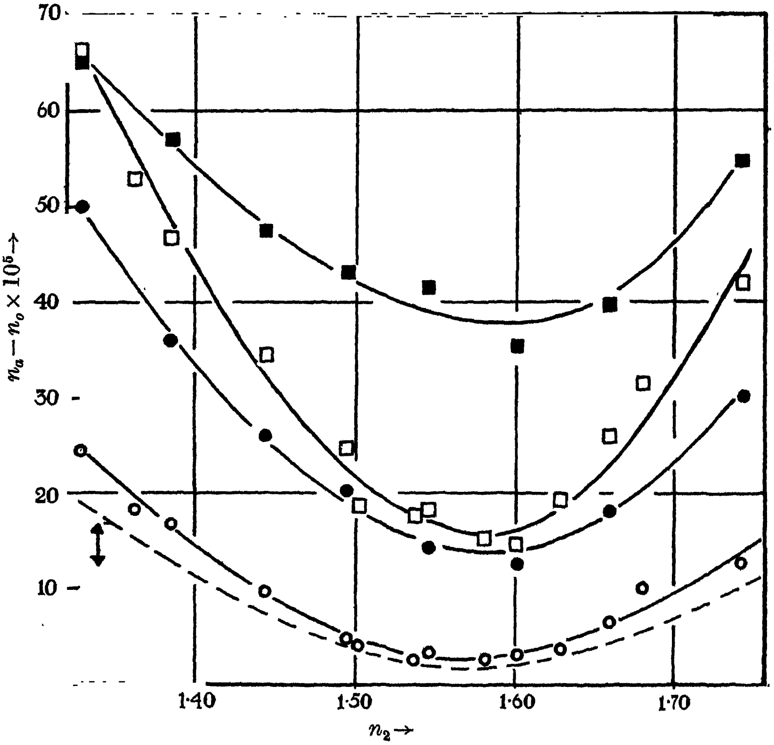

Fig. 2—Curves showing dependence of axis cylinder birefringence $n_a - n_o$ on refractive index, n_2, of the immersion medium. The various preparations are: ○ $HgCl_2$-fixed axon corrected for the sheath; ● isolated axis-cylinder strand obtained from an axon fixed in 35 % alcohol; □ similar to ● but from different axon; and ■ air-dried strand from same preparation as ●. The data are tabulated in Table I. The significance of the dotted line and arrow is shown on p. 514.

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* It is difficult to obtain accurate estimates of the relative shrinkages which have occurred in the preparations of fig. 2 without information as to the length changes as well as the diameter variations occurring during the various manipulations employed. If, however, we neglect the relatively unimportant length changes, it follows that D_1/D_2 squared represents the ratio of volumes occupied by a given amount of axis-cylinder solid in the states having diameters D_1 and D_2. Hence, in the above preparations the solids from unit volumes of the fresh axons occupy the volumes 0·81, 0·33, 0·20, and 0·08 after the shrinkage. It has been observed that, depending upon conditions, variable length changes occur upon air-drying of alcohol-

apparent: (1) that the minima of the curves are raised as shrinkage progresses; (2) that the steepness of the rise of the form curves from the minima increases with small decreases in diameter and then sinks again to become rather shallow in the highly shrunken case. This is just the behaviour to be expected of a shrinking mixed body with an oriented anisotropic component, as will now be shown.

As stated in the introduction, the optic axis of the axoplasm parallels the length of the fibre. This has been confirmed by us from cross-sections of squid axons. Thus the form birefringence of the present material is positive and uniaxial and hence it is similar to that which would result from cylindrical rodlets or fibrils oriented with their long axes paralleling the axon length. Wiener's (1912) formula for rodlet form birefringence may be written in approximate form:*

$$(n_a - n_o)_f = \frac{\delta_1\delta_2(n_1^2 - n_2^2)^2}{2[(\delta_1 + 1)\,n_2^2 + \delta_2 n_1^2]\,(\delta_1 n_1 + \delta_1 n_2)}, \tag{1}$$

fixed axons, and though in some cases it may be required that D_1/D_2 be raised to a power as high as 2·5, this is of minor importance except in the case of the air-dried preparation. The figure 0·08 given for this strand is, however, in good agreement with what is known regarding the composition of invertebrate nerve. Thus, by using the data of Young (1936) for relative proportions of axis cylinder in cephalopod nerves; the density 1·35 of dried gelatine as typical of dried protein; analysis of crustacean nerves (Schmitt and Bear, unpublished), which Young has found to be similar in structural composition to cephalopod nerves, for the estimation of relative amounts of axis-cylinder protein, other protein, lipoid, and ash; and 0·08 as the relative volumes of protein in the axoplasm, it can be calculated that typical whole invertebrate nerve has a composition by weight expressed roughly as follows: 8 % axis cylinder protein, 4 % other protein (connective tissue, etc.), 2 % lipoid, 3 % ash, leaving about 83 % for water content. The latter figure is almost exactly that observed in invertebrate nerve as typified by lobster leg and claw nerves. According to these figures the axoplasm can be regarded as being 10–11 % protein by weight. It is possible, however, that in these calculations some error in distribution of protein between the axis cylinder and the other regions of the nerve has been made. A lower limit for the relative volume of axis-cylinder protein can certainly be set at 4 %, however, and this is sufficient for the present purposes, since this is still far above the 0·3–0·4 % indicated later as being effective in determining the birefringence of fresh axoplasm.

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* This form of the expression for the rodlet form birefringence is more convenient than the usual one which expresses $n_a^2 - n_o^2$. It is valid to the extent that

$$(n_a^2 - n_o^2) = (n_a - n_o)\,(n_a + n_o) \cong (n_a - n_o)\,2n_a$$

and that

$$n_a^2 = \delta_1 n_1^2 + \delta_2 n_2^2 \cong (\delta_1 n_1 + \delta_2 n_2)^2.$$

These approximations are particularly good in the neighbourhood of the minimum where n_2 is nearly equal to n_1 and where $n_a - n_o$ is small. Though the optical phenomena could be at least qualitatively explained by assumption of platelets or ribbons with normals to flat faces oriented at random in planes perpendicular to the axon axis, exact expressions for these cases are not known nor would the accuracy of the experimental results or the conclusions to be drawn allow or require such a refinement at the present time.

in which n_1 is the refractive index of the anisotropic micelles involved, n_2 that of the immersion medium, δ_1 and δ_2 are the relative volumes of micelles and immersion medium, respectively, and n_a and n_o the respective extraordinary and ordinary refractive indices of the mixed body, the difference being the experimentally measured form birefringence. Since n_1 is fixed in the present cases and $\delta_1+\delta_2=1$, it follows that $(n_a-n_o)_f$ is expressed in equation (1) as a function of the independent variables n_2 and δ_1 (or δ_2). The numerator of (1) is most effective in determining the shape of the form curve, hence it immediately follows that for a given $(n_1^2-n_2^2)$ the magnitude of $(n_a-n_0)_f$ will be dependent largely upon $\delta_1\delta_2$, or that the steepness of a curve with constant $\delta_1\delta_2$ will be a reflection of the magnitude of $\delta_1\delta_2$. The value of $\delta_1\delta_2$ is small for highly expanded or highly contracted mixed bodies, and between these limits this product has maximum value when $\delta_1=\delta_2$. Thus we have the explanation of rise to maximum steepness of the form curve with initial shrinkage of the axon and the return to shallow curves for continued shrinkage.

In the case of the minima, however, we have

$$(n_a-n_o)_i=\delta_1(n_{a1}-n_{o1}), \tag{2}$$

where $(n_a-n_o)_i$ is the intrinsic birefringence and n_{a1} and n_{o1} are the respective extraordinary and ordinary refractive indices of the anisotropic micelles. Hence it is apparent that shrinkage, since it continually increases δ_1 while $(n_{a1}-n_{o1})$ remains essentially constant, should continually increase the birefringence at the minimum, as is actually the case.

From these considerations one immediately sees that the positions of the birefringence of the fresh axons as placed in fig. 2 are as would be expected when it is recalled that the fresh axons represent an expanded state, fixation always resulting in a certain amount of shrinking. It is possible by empirical methods to extrapolate from the curves for fixed axis cylinder to a similar curve for the fresh axon. One such result is shown in fig. 2 by the dotted line. The agreement between this curve at $n_2=1{\cdot}34$ and the measured fresh-axon birefringence (double arrow on fig. 2) is as good as could be expected from the uncertainties arising from the various connective tissue corrections used with the $HgCl_2$-fixed material and the fresh fibres. It seems reasonable, therefore, to assume that in living, as in fixed axoplasm, micelles with both form and intrinsic anisotropies are present, though, of course, in the normal condition the optical effects caused by the micellar shape predominate more strikingly over those arising from intramicellar anisotropy than is the case in the shrunken axons. The empirical method of extrapolation is not given in detail since it has little theoretical significance.

Since from the experimental data values for n_1, n_2 and $(n_a-n_o)_f$ are known for use in equation (1), it is possible to calculate "apparent" values of $\delta_1\delta_2$ for the curves of fig. 2. To do this roughly we consider the observed birefringences at $n_2=1{\cdot}33$ and let n_1, as indicated by the positions of the minima, be approximately 1·58. The denominator in equation (1) is almost independent of the relative volumes, being approximately 11 in the cases under consideration for any permissible values of δ_1 and δ_2, so that from the data of the top line of Table I (after subtracting the intrinsic birefringence) it is a simple matter to show that the apparent values of $\delta_1\delta_2$ for the four curves of fig. 2, going from the lowest to the uppermost, are about 0·0049, 0·0071, 0·011 and 0·0060. The striking fact which emerges is the extreme smallness of the apparent $\delta_1\delta_2$ throughout the wide range of shrinkage. This product can theoretically attain values as high as 0·25 (when $\delta_1=\delta_2=0{\cdot}5$), but for no curve does it appear to be as high as a twentieth of this value. Furthermore, since at low δ_1 we have $\delta_1\delta_2\cong\delta_1$, it is obvious that for the fixed material most near to the fresh axon state, i.e. the $HgCl_2$-fixed preparation, the relative volume of anisotropic material responsible for the form birefringence would be a fraction of a percentage which is much too low a figure for the protein content of the axis cylinder. From similar considerations of fresh-axon birefringence this figure would be even lower in the living material (0·3–0·4 %).

We must conclude, therefore, that in axoplasm we are not dealing with the ideal rodlet mixed body for which equation (1) is designed. This, however, does not invalidate use of the equation to explain qualitatively the reasons for the varying steepnesses of the form birefringence curves, for it is readily seen that, even for non-ideal systems, at the limiting cases of $\delta_1=0$ or $\delta_2=0$ the form birefringence will disappear and between these the form effects will reach a maximum.

DISCUSSION

The present experiments provide the first definite evidence for the presence in fresh axoplasm of micelles having anisodiametric shapes as well as intrinsic crystalloidal structure. Perhaps of even greater importance is the demonstration that although the data from the immersion experiments give curves similar qualitatively to those to be expected of an ideal two-component rodlet-mixed body of Wiener, the birefringence of fresh axoplasm ($1{\cdot}5\times10^{-4}$) is much too weak to result from a simple system of this type (involving *all* the protein in the axoplasm). Though the establishment of this fact is of primary importance, it follows that an interpretation of the ultrastructure of fresh axoplasm must be arrived at from the various possibilities still left open by the present data, largely by a consideration of supplementary information from X-ray diffraction, chemical and histological data.

Before discussing the bearing of supplementary data, the simplest possible reasons for the inapplicability of equation (1) are enumerated as follows:

(1) The shape of the micelles may depart widely from the limiting forms of Wiener, i.e. perfect rods or platelets, for which the theory was designed.

(2) The alignment of the micelles, though symmetrical about the optic axis, may be very poor.

(3) The micellar anisotropy may be the property of a species of micelle which occupies but a very small proportion of the volume, the great bulk of the axoplasm being comprised of unoriented or essentially isotropic, nearly spherical micelles.

(4) Other more complicated possibilities might be suggested. For example, the optical properties may be the result of two sets of micelles of opposing optical characteristics. The preponderant species might consist of rodlets with axes paralleling the length of the axon, the opposing species possessing platelet form with face normals also in the longitudinal direction.

The discussion of these possibilities is most profitable from the standpoint of two alternative hypotheses: the optical properties are determined either (A) by the major fraction of the protein material, or (B) by a small fraction of well oriented anisotropic material. According to hypothesis A, possibilities 1, 2, 4, or combinations of these, would be necessary to explain the weak form and intrinsic birefringence, while hypothesis B involves principally possibility 3.

The histological and optical data appear to be reconcilable only by the assumption of hypothesis B. It is difficult, for example, to account for the ease with which the axon may become filled with extremely well oriented fibrils, as a result of relatively slight imbalances, except on the supposition that some fraction of the protein material is rather well oriented. That fibril formation in many instances does not involve alignment of micelles, or indeed any considerable changes in their orientations would seem to follow from the absence of any great discontinuity between the birefringences of the fresh and fixed axons of fig. 2. It must be borne in mind, however, that the experiments in which fibrillation was demonstrated in the absence of accompanying birefringence could be interpreted as indicating that attempts to correlate visible fibrillation and birefringence may be unwarranted. It would seem more likely, however, that these effects may result from the destructive nature of the reagents employed (urea and alkali), as has been mentioned. In the majority of cases, fibrillation and birefringence appear to be related, and particularly is this apparent in the case of the movable fibrils produced by compression of an axon.

The lack of X-ray diffraction evidence for orientation in the axis cylinder (Boehm 1933; Schmitt, Bear and Clark 1935) cannot be said to forbid assumption of hypothesis B, since the amount of the well oriented component may be too small to be detected against a background of isotropic protein material, particularly if the organized micelles are so minute as to present too few diffracting planes for the production of well defined interferences. The diffraction data do, however, definitely point to the discarding of possibilities of the type 4, at least in so far as they could be employed in connexion with hypothesis A, since the great amount of orientation which might then be expected, without accompanying increased birefringence, is not observed.

The only reason for considering possibility 4 at all comes from evidence obtained in the work on the protein of the axis cylinder to be described in the following paper. There it is indicated that a small fraction of this protein complex is of phosphorus-containing nature, similar in many respects to nucleic acid. Schmidt (1936) has found that in structures containing nucleic acid and protein (chromosomes) the magnitude and sign of birefringence is dependent on the relative proportions of these two components, the former favouring a negative character while the predominance of the latter leads to positive birefringence. Small amounts of nucleic acid can have an important effect, though it appears that the nucleic acid contributions are largely of *intrinsic* nature. Since the problem in the case of nerve demands low *form* as well as intrinsic birefringence, and since the nucleic or pseudo-nucleic acid content of axoplasm is extremely small, it would seem that if possibility 4 plays any role at all in the determination of nerve birefringence it is a minor one.

It is of interest to see whether application of hypothesis B to the optical data leads to reasonable conclusions. It can be shown that application of Wiener's theory to a three component system composed of isotropic particles, anisotropic rodlets, and immersion fluid leads to an expression which is approximately equal to equation (1) when the total dispersed material occupies volume small in proportion to the total volume. In such a case δ_1 becomes the relative volume of the anisotropic component, hence in the case of the $HgCl_2$-fixed axon according to hypothesis B the relation $\delta_1\delta_2 \cong \delta_1 = 0{\cdot}005$, previously calculated according to equation (1) and the observed form birefringence, permits approximate determination of the amount of oriented material present. Dividing the intrinsic birefringence, $2{\cdot}5\times10^{-5}$, by this δ_1 value leads to 0·005 for the approximate micellar intrinsic birefringence (equation (2)). This figure represents a reasonable value for a protein micelle (Weber 1935, found 0·008 for myosin). Turning to the other extreme of the optical data, one finds, again employing equation (2) (which is quite accurate for *any* system containing a single well oriented component) and using 0·005 for the micellar birefringence and $3{\cdot}5\times10^{-4}$ for the intrinsic birefringence of the air-dried preparation, that 7 % of the volume of this preparation is occupied by the anisotropic component. Since in a previous footnote it was indicated that the air-dried preparation has been shrunk to about 4–8 % of the original fresh axon volume, it follows that roughly 0·3–0·6 % of the fresh axon volume would be occupied by the anisotropic component. These figures are not inconsistent with the corresponding values indicated by the fresh axon birefringence (0·3–0·4 %) as mentioned previously. While these calculations are probably not to be taken too literally, they do indicate the reasonable character of hypothesis B as well as the self-consistency of the data.

SUMMARY

The use of the giant axons of the squid, *Loligo pealii*, has permitted for the first time a quantitative approach to an analysis of the factors which produce the birefringence of nerve axoplasm. By employing the immersion technique it has been demonstrated that protein micelles with both form and intrinsic anisotropies are responsible for the optical properties of fixed axons. Rough extrapolation from the observations on axons fixed in a number of ways, or removed from their sheaths and shrunken by varying amounts, shows that the same effects that operate in the fixed material undoubtedly are present in the fresh axoplasm.

It has been demonstrated that the birefringence of fresh axoplasm ($1{\cdot}5\times10^{-4}$) is too low to result from the entire protein content, if this is assumed to consist of a single species of well oriented rodlet micelles. Since several possible reasons for the weak birefringence exist, no unique solution for the ultrastructure can be reached from optical data alone. Because of the demonstrated relation between birefringence and fibril formation, it has been concluded that the optical properties are determined by a relatively small well oriented fraction of its protein complex. This view is not inconsistent with X-ray diffraction and chemical information obtained from similar material.

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DESCRIPTION OF PLATE 24

FIGS. 3–6 refer to this paper. FIGS. 7–9 to that which follows.

FIG. 3—An isolated giant fibre from the hindermost stellar nerve of the squid, *Loligo pealii*, after treatment with 35 % alcohol. The central strand (*ax*) consists of shrunken axis cylinder, the investing layers of connective tissue, etc., being left as a hollow tube (*s*) with practically the same diameter as the original fibre. Magnification 57 ×.

FIG. 4—The same preparation as in fig. 3 viewed between crossed Nicol prisms. A rotating $\lambda/20$ plate compensator is set to extinction of the region at the centre of the tube of sheath material (s_1) which is free of axis cylinder. This portion is somewhat blacker than the field, indicating its negative character, while the axis cylinder strand (*ax*) and the edges of the tube (s_2) show brightness because they are positive with respect to fibre length. Magnification 70 ×.

FIG. 5—The axis-cylinder strand of fig. 3 after removal from its investing layers. Magnification 57 ×.

FIG. 6—The same strand after air drying. Magnification 57 ×.

(Figures in suceeding paper).

FIG. 7—Transverse section of axis-cylinder extrudate from a giant axon. The extrusion was made into distilled water and the extrudate immediately fixed in 5 % $HgCl_2$. Sections were stained with haematoxylin and eosin. The thin dark edge probably represents the "periaxial membrane". Magnification 320 ×.

FIG. 8—Basophilic bodies in the giant axon of the squid as found within the stellate ganglion. The preparation was fixed by injection of Regaud's fluid and stained with Masson's stain. Magnification 290 ×.

FIG. 9—Similar to fig. 8, except that the region photographed was outside the ganglion and Flemming's fluid was used for fixation (without acetic), the Azan technique for staining. Magnification 290 ×.

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Investigations on the Protein Constituents of Nerve Axoplasm

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Introduction

Studies made in the past on the proteins of nervous tissue have been carried out almost exclusively with the large masses of material available in the brain and spinal cord of vertebrates. Because of the great complexity of such material it has been impossible to determine the position of the proteins in the various histological components of the neuron. It is uncertain, for example, which of the proteins are characteristic of the axis cylinder on the one hand and of such structures as chromatin or Nissl substance of the cell body on the other. Recently, Schmitt and Bear (1935), realizing this situation, began studies of the proteins of crustacean peripheral nerve. This tissue was chosen both because it consists predominantly of axoplasm and because its low lipoid content allows direct extraction of the proteins without preliminary desiccation or lipoid removal. It was found that the largest part (about 65 %) of the protein of the entire nerve (including connective tissue) is extracted in neutral salt solutions and that the properties of this soluble protein are in many respects similar to those described by previous investigators working with mammalian central nervous system, the most reliable results being those of McGregor (1917). Though the amount of this fraction agrees roughly with estimates of the relative amount of axis cylinder in crustacean nerve (Young 1936), it was still not certain that all the components of the conjugated protein present could be considered to be derived solely from axoplasmic protein, for it was demonstrated that in lobster nerve an alkali-soluble fraction similar to the "neurostromin" of Danilevsky (1891) and Shkarin (1902) is also present.

The axoplasm of the giant nerve fibres of *Loligo* may be obtained merely by extrusion from a cut end. In this way it is possible to obtain the axoplasmic complex uncontaminated by foreign material. The present paper contains a preliminary account of the properties of the proteins of the axon as revealed by study with a special microtechnique of this extruded axoplasm.

METHODS

Pure axoplasm was obtained as follows. The ligated giant fibre, teased free of most of the adhering connective tissue and small axons, and washed in sea water or distilled water, was placed on a depression slide so that the well, containing an appropriate solution, lay under the middle portion of the fibre. The axon was then cut in the centre and the axoplasm extruded into the solution from each half by compressing the fibre at the closed end with the edge of bent forceps and moving the latter down towards the open end. Immediately after extrusion the remaining sheath material was removed to avoid contamination and mechanical disturbance of the extruded material. Fibres which had been dipped in distilled water for a few seconds before extrusion, showed a striking turgor, becoming quite stiff. Cutting of a fibre in this condition usually resulted in self-extrusion of the axoplasm on account of the internal pressure arising from swelling shortening of the circular connective tissue fibres, the swelling of the colloids of the axoplasm itself perhaps also assisting.

After thorough mixing of the axoplasm with the medium into which it was extruded, the contents of the depression slide were removed by pipette to a small tube (4 × 60 mm.), in which centrifuging and chemical treatment could be carried out. Material obtained from 4 to 8 axons, each being 5–7 cm. in length and 0·4–0·6 mm. in diameter, sufficed for most purposes.

EXPERIMENTAL RESULTS

1—*Experiments on Intact Axons*

At the beginning of the experiments an attempt was made to determine whether the materials in the axis cylinder of intact fibres could be studied by observations of their reaction to treatment with reagents. Left in sea water or neutral isotonic salt solution for a number of hours, intact ligated fibres degenerate in a typical manner. The axoplasm first develops fibrillation, and then there is a breakdown of the fibrils into rows of granules. This linear arrangement of the granules next disappears, and eventually most of the granulation itself disappears leaving the central region of the fibre almost clear. A small residue, however, of easily movable whitish granules invariably remains, as can best be seen against a dark field.

In distilled water the series of events is similar but much slower than in sea water, whereas in alkaline media (isotonic salt solutions buffered to *p*H 10–14) the reaction is much faster, being over in a matter of minutes rather than hours. The whitish residual granules, however, resist solution even in molar NaOH. In acid the axoplasm is precipitated to a dense white mass immediately; the nature of this precipitate will become clear below.

2—*Extrusion of Axoplasm into Reagents*

In a successful extrusion one obtains a rod of material several centimetres long and similar in shape to the fibre from which it was extruded. The physical and optical properties of the extrudates vary considerably, depending on the medium into which the extrusion was made. The rate of disintegration is influenced by at least three factors: (1) salt concentration, (2) *p*H of the surrounding solution, and (3) the restraining action of a thin, chemically stable membrane often seen to enclose the extrudate. In sea water or in isotonic salt solutions the extrudate immediately begins to disintegrate, swelling and going into solution. The positive birefringence, which at the moment of extrusion is only a fraction of that of the intact axon, begins to fade and disappears after 10–20 min. In distilled water, on the other hand, the extrudate sets to an opalescent gel which retains its positive birefringence much longer (30–40 min.). Alkaline media bring about rapid disappearance of birefringence (few seconds) and of the original shape of the extrudate. Acid media coagulate the material to an insoluble, rather opaque cylinder.

In favourable extrusions it has been possible to distinguish a thin membrane surrounding the extrudate (fig. 7, Plate 24 of previous paper). The presence of this membrane is manifested most strikingly at the ends of the extrudate, where the fibrillated axoplasm extends out beyond the membrane in a fan-shaped configuration. Disintegration of the extrudate takes place most rapidly in portions denuded of the membrane, which in badly done extrusions may be seen trailing behind as a delicate veil at the end last extruded.

Because of its thinness and tenuous nature this membrane cannot belong to any portion of the axon investments previously described but appears to be a part of the axon itself. This is confirmed by the fact that all attempts to find nuclei in or near it by methylene blue staining have been negative. Though it is difficult to decide whether this membrane really exists in the normal condition as a preformed periaxial sheath or is the result of exposing the axon to a medium of foreign chemical composition, the facts that it is occasionally found trailing the central material and that the underlying axoplasm will still flow out from it without the formation of a new film suggest that it may not be an artifact.

After complete disintegration of an extrudate there remain only the thin veil-like periaxial membrane and numerous small granules of insoluble residue; these are quite resistant to alkaline attack, remaining even after hours in 0·1–1·0 M NaOH. By far the larger proportion of the colloid of the axoplasm goes into apparent solution, as can be confirmed by acid treatment, which results in considerable precipitation.

3—*Chemical Properties of the Axoplasmic Protein*

In the following paragraphs the observations are described with reference to the properties of the soluble portions only of the colloids of the axon. For the present the small amount of insoluble granules and membranes referred to above is not considered, these being removed by centrifuging.

Axoplasm, extruded into sea water or neutral isotonic KCl or NaCl, dissolves rather rapidly, and one soon obtains a slightly opalescent solution. If an equal volume of axoplasm is extruded into the same amount of distilled water, solution is fairly rapid at first, but soon slows down and stops. If the volumes of dissolving fluid in the two cases are not too large (about four drops of fluid for the material of four 5–7 cm. axons), it can thus be demonstrated that the axoplasm is more soluble in salt solution than in distilled water, since in the latter saturation is quickly reached. This behaviour is suggestive of the solubility properties of a globulin, but addition of a relatively small amount of distilled water to the portions remaining undissolved in the original distilled water extrusion shows a considerable solubility even in the absence of salt. Moreover, it is not possible to reprecipitate any of the protein from salt solution by addition of water unless the original concentration of protein is high and the dilution with water not too great.

Slight acidification of the protein solution with acetic acid (to *p*H 4·5–5·0) results in a marked increase in opalescence or an actual precipitation. With low salt concentrations precipitation occurs immediately in fairly copious amounts, whereas it is difficult to obtain a satisfactory separation of the protein upon acidification of a solution containing considerable salt. Heat coagulation, however, in the latter case suffices to bring down the precipitate in an amount roughly judged to be equal to that obtained from the distilled water solutions of equal protein concentration.

Addition of a small amount of alkali to a saturated distilled water extrusion greatly increases the amount of axoplasm which the water can dissolve. Similarly the precipitate produced by acidification can be redissolved in faintly alkaline solution (pH < 12), though if the precipitate has been allowed to stand for any time (minutes) in contact with acid, variable amounts will be left undissolved, these requiring stronger alkali (pH > 12), and sometimes warming, to cause resolution. The protein can be reprecipitated from alkaline solution by the addition of acid, but in the case of solutions of the above acid-treated material neutralization seems to suffice. The latter thus resembles acid metaprotein in its properties.

The protein originally present in the axon has at least two components. If the axoplasm is extruded into or treated with alkali the solution is complete, except for the small amount of insoluble residue always present, and the solution can be made quite clear by centrifuging. On standing a short time in alkali, one may notice a slow precipitation of a flocculent, almost invisible component, which is particularly plentiful when the solution is heated on a water-bath. If, after the alkali treatment (even though no great amount of this basic component has been formed), the acid-precipitable component be removed (by making pH *ca.* 5, heating for several minutes, and centrifuging) it is possible to obtain more alkali-insoluble protein by adjusting the pH of the solution to 11 or 12. This seems to be the optimum precipitation range for this material, as can be shown by over-alkalinizing the solution, centrifuging off the precipitate, and returning the centrifugate to this pH range, whereupon further precipitation occurs. The amount of this alkali-insoluble precipitate obtainable from a given preparation seems to be limited and represents an appreciable fraction of the total protein present, though it is always less than the amount of acid-insoluble material found in the same sample. The basic component, which seems to be similar to a histone or protamine, can be dissolved in dilute acid and reprecipitated in alkali, though long standing (hours) in base produces a relatively insoluble alkali metaprotein. It is possible that this fraction may be related to the small proportion of alkali-resistant particles found in the disintegrating intact axons and extrudates as described above, though we have devoted no attention to this question as yet.

Fresh solutions of extrudate may be almost cleared of protein by half-saturation with $(NH_4)_2SO_4$ or Na_2SO_4, or by slight acidification combined with heat coagulation, but there always remains behind a small amount of protein requiring higher salt concentration or more efficient protein precipitants, such as trichloracetic and picric acids, for its removal. This residual protein will include the alkali-insoluble component only if the original extruded material has been previously treated with alkali. In a few cases by careful buffering with moderately strong concentrations of sodium acetate (10%) and acetic acid to a *p*H in the neighbourhood of 5, followed by heat coagulation and centrifuging, a solution was obtained containing a small amount of a substance precipitable by addition of mineral acid (HCl to a concentration of 0·1 N). This solid was soluble again in distilled water and alkali, being capable of reprecipitation and resolution several times without apparent changes in solubility. A small precipitate was also obtained after brief treatment of a fresh extrudate solution with alkali, precipitation with acetic and picric acids, centrifuging and addition of an excess of alcohol and acid to the centrifugate.

These experiments, as well as the solubility properties of the main bulk of the protein of the axis cylinder, suggest the presence of a nucleic or pseudo-nucleic acid. From the points to be brought out below it will be clear that any such phosphorus-containing compound could be expected in but very small amounts at best, hence it is not surprising that with the extremely small amount of material available, the results have been inconclusive.

The presence of phosphorus in the precipitated "nucleo-protein" is easily demonstrated by the molybdate reaction after decomposition with nitric and sulphuric acids. The origin of this phosphorus is not certain, however, because of the presence of a small amount of phosphatid. The latter was detected by the myelin forms produced by wetting the solids which remained after evaporating a benzene extract of dried extrudates. Because of the small quantities of material, preliminary removal of the phosphatids is impracticable.

More positive proof for the presence of nucleic acid would be the isolation of purine bases. This has been attempted from material obtained by acid hydrolysis of whole extrudate or of appropriate fractions, but the results have been inconclusive. Acid hydrolysis of fractions which would contain the nucleic acid did yield a positive reaction for desoxysugar when tested by the method of Dische (1931, blue colour with diphenylamine). This would be good evidence for the presence of animal nucleic acid or nucleotides were it not for the fact that the rather lengthy hydrolysis required (more than 30 min.) and the faint colours obtained make the conclusion somewhat uncertain.

Another test which depends on the presence of desoxysugars is the Feulgen nucleal reaction. Applied to longitudinal sections of fixed axons this test was entirely negative for the axis cylinder region, although the nuclei of the connective tissue layers gave very positive reactions. Negative nucleal reactions may mean nothing, however, for Caspersson (1936) has shown that the presence of a relatively small amount of protein serves to mask the nucleic acid completely for the purpose of this test. The difficulties of these attempts for nerve protein are emphasized by the low phosphorus figures obtained by Levene (1899) and Shkarin (1902).

Though the demonstration of a nucleic acid in the squid axon cannot be said to be sufficiently definite at present, the following evidence from other nervous tissues can be cited in corroboration of several of the results described above. The protein solutions obtained by treatment of fresh macerated lobster nerve or of dried lipoid-extracted cow spinal cord with neutral salt solutions or water resemble in practically every detail the squid axis-cylinder preparations. Because of the larger quantities available in the extracts of these materials, experiments have been made to aid the characterization of the nucleic-acid-like fraction. Preliminary results show a somewhat more positive Dische test in the case of the lobster strong-acid-insoluble fraction, while with the similar cow spinal cord portion this test is typical in every way of that given by animal nucleic acid. Furthermore, it has been shown that in both of these cases this fraction contains non-lipoid organic phosphorus, which is split off by hydrolysis in acid from a base or bases that form silver salts upon treatment with ammoniacal silver. The strong-acid-insoluble fraction, as usually obtained, is still rich in protein, but even after several purifications quantitative estimation indicates that the P-content is low, agreeing with the result of Levene on brain nucleic acid (3 % P as compared to the 10 % expected for true nucleic acid).

Highly suggestive of the presence of an acidic component in the squid axoplasm is the presence of basophilic rodlets observed in material fixed in Flemming's fluid (without acetic acid), Regaud's fluid, chromic acid, or Bouin's fluid, and stained with iron haematoxylin or azocarmine. They vary in size from particles of a micron or less in length to rods of considerable size (figs. 8 and 9, Plate 24 of previous paper). They are always oriented lengthwise in the axon. In some cases they resemble continuations of single fibrils or aggregates of fibrils, suggesting a close relationship between the basophilic substance and the protein fibrils. A further interesting fact is that these basophilic bodies are most prominent close to the cell bodies of the axons. Thus in the stellate ganglion they are often seen just before the fibres unite at the base of the giant fibre lobe, but the more peripheral parts of the axon rarely show them. They are also to be found in the proximal part of the second order fibres in the central nervous system, whereas the more distal parts of the first and second order axons are devoid of them. Moreover, the giant cells of the first order axons contain Nissl substance in the form of rods which grade off into fibrillar threads not unlike the basophilic filaments. Whether the Nissl substance and the basophilic bodies grade down with respect to the "nucleic" acid until in the peripheral axon the amount is too small to be readily detected remains to be determined by more extensive microchemical experiments.

DISCUSSION

The fact that the description of the solubilities and general reaction characteristics of the principal squid axon protein described above applies almost word for word to the analogous fraction obtained from such widely different sources as lobster claw nerve and mammalian (cow or sheep) brain or spinal cord, is strongly suggestive that this protein is characteristic of neuroplasm generally, just as myosin is typical of contractile tissue. Since it is probably the principal protein of the central as well as of the peripheral nervous system its presence in considerable amounts in the nerve cell is suggested. It would, indeed, seem reasonable to associate this protein with the material responsible for the fibrillar structures that can be seen so abundantly in many types of nerve cells which have been subjected to the histological techniques suitable for making these structures apparent. This inference receives support from the fact that many of the qualitative observations on the birefringent properties described in the preceding paper for the axis cylinders of squid axons apply equally well to certain regions of the protoplasm of vertebrate spinal ganglion cells (Chinn, unpublished).

Both for convenience and to emphasize its apparently universal distribution in nervous tissue we propose to apply the name *neuronin* to this protein complex. None of the previously used names is appropriate. Thus the "neuroglobulin" of Danilevsky (1891) and Halliburton (1894) is inappropriate, since McGregor (1917) has shown that the protein is not a globulin. "Cerebronucleoproteid" (Levene 1899) is not sufficiently general, for it implies a localization in the nervous system which we know now is too limited. Furthermore, it is advisable at the present time to avoid specific chemical names, such as might bear the prefix "nucleo-," for example.

A significant fact which emerges from the present description of the properties of neuronin and from the results of the preceding paper on the optics of the axis-cylinder is the intimate connexion between many morphological, physical, and chemical properties of the axis cylinder. That neuronin represents the largest part, if not all, of the material of the neurofibrils follows from the great abundance of the fibrils, which at times fill the entire axon, and from the fact that neuronin represents the great bulk of the protein of the axoplasm. The chemical properties of neuronin show further that it is a complex of several components, a conclusion which was also indicated independently by the optical and histological data. It is too early, however, to attempt to relate the anisotropic component, which has been taken to be responsible for the birefringence and fibril formation, to any definite chemical component of neuronin. Indeed the chemical and optical divisions of neuronin might conceivably be along quite different lines.

That the state of neuronin in the extracted dissolved condition differs from that obtaining in the living axis cylinder is obvious. For example, the birefringence of the living axis cylinder shows oriented organization which, though small, could not exist in a true liquid at rest. Nevertheless, observations on the effect of salt concentration and *p*H on neuronin solubility *in vitro* are confirmed by observations made on the intact axon. These environmental factors may have an important bearing on the state of aggregation of the axoplasm at rest and in activity.

SUMMARY

It has been possible, by means of a suitable microtechnique applied to the giant axons of the squid, *Loligo*, to isolate uncontaminated axoplasm for an investigation of the chemical properties of the protein components of this substance. Information gained in this way has pointed to the essential similarity between the protein in the giant axon of the squid and that obtained from neutral extracts of a wide variety of nervous tissues, i.e. from lobster peripheral nerve and lipoid-extracted mammalian central nervous system. Thus this protein complex, neuronin, seems to be characteristic of neuroplasm generally, as found both in the axons and the cell bodies of neurons.

Neuronin solubility properties are those of a nucleo- or pseudo-nucleoprotein. It is not very stable, in dilute acid going over into a relatively insoluble metaprotein. After brief standing in alkali a fraction is split off which resembles a histone or protamine in solubility characteristics. Under proper conditions, such as heat coagulation in the presence of dilute acid and high salt concentration, the bulk of the neuronin protein can be removed from a phosphorus-containing nucleic-acid-like fraction. The definite characterization of the latter part of the neuronin complex has not been made as yet because of the small proportion present.

The maintenance of the normal state of aggregation of the neuronin complex apparently depends to a large extent on the presence of dissolved

salts and on the *p*H, which therefore may be factors of considerable importance in determining the state of aggregation of the neuronin micelles during normal functioning of the axon.

In addition to the soluble portion of the axoplasm a small amount of insoluble residue is also found. The insoluble portions are rather resistant to chemical attack and have not as yet been investigated to any extent.

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INDEX TO VOLUME CXXIII (B)

Aerobic respiration in barley seedlings and its relation to growth (Barnell), 321.
Alimentary secretions of intact animals, effect of posterior pituitary extract on (Cutting, Dodds, Noble and Williams), 27.
Ashby (E.) Heterosis and the inheritance of quantitative characters, 431.
Atkins (W. R. G.) *See* Poole and Atkins.

Bachmann (W. E.), Cook (J. W.), Dansi (A.), de Worms (C. G. M.), Haslewood (G. A. D.), Hewett (C. L.) and Robinson (Mrs. A. M.) The production of cancer by pure hydrocarbons, IV, 343.
Bacteria, action of radiations on (Lea, Haines and Coulson), 1.
Barnell (H. R.) Analytic studies in plant respiration: VII—Aerobic respiration in barley seedlings and its relation to growth and carbohydrate supply, 321.
Bawden (F. C.) and Pirie (N. W.) The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus, 274.
Bear (R. S.), Schmitt (F. O.) and Young (J. Z.) Investigations on the protein constituents of nerve axoplasm, 520.
Bear (R. S.), Schmitt (F. O.) and Young (J. Z.) The sheath components of the giant nerve fibres of the squid, 496.
Bear (R. S.), Schmitt (F. O.) and Young (J. Z.) The ultrastructure of nerve axoplasm, 505.
Bell (Julia) and Haldane (J. B. S.) The linkage between the genes for colour-blindness and haemophilia in man, 119.
Bhimachar (B. S.) A study of the medulla oblongata of Cyprinodont fishes with special reference to their feeding habits, 59.
Biological material, quantitative spectrographic analysis of (Foster and Horton), 422.
Bose (S. R.) and Sarkar (S. N.) Enzymes of some wood-rotting *Polypores*, 193.
Brooks (J.) The action of nitrite on haemoglobin in the absence of oxygen, 368.
Burrows (H.), Mayneord (W. V.) and Roberts (J. E.) Neoplasia following the application of X-rays to inflammatory lesions, 213.

Cancer, production of by pure hydrocarbons (Bachmann and others), 343.
Clark (W. E. Le Gros) The connexions of the arcuate nucleus of the thalamus, 166.
Colour-blindness and haemophilia in man, linkage between the genes of (Bell and Haldane), 119.
Cook (J. W.) *See* Bachmann and others.
Coulson (C. A.) *See* Lea, Haines and Coulson.
Crawford (B. H.) The change of visual sensitivity with time, 69.
Cutting (W. C.), Dodds (E. C.), Noble (R. L.) and Williams (P. C.) Pituitary control of alimentary blood flow and secretion, 27, 39, 49.

Dansi (A.) *See* Bachmann and others.
de Worms (C. G. M.) *See* Bachmann and others.

Discussion on the transmission of excitation in living material (Pantin), 397.
Dodds (E. C.), Noble (R. L.), Scarff (R. W.) and Williams (P. C.) Pituitary control of alimentary blood flow and secretion. Changes in the stomach produced by the administration of posterior pituitary extract, 22.
Dodds (E. C.) *See* Cutting, Dodds, Noble and Williams.

Ear, human, response of to audio-frequency electrical stimulation (Hallpike and Hartridge), 177.
Equation, differential, graphical solution of in relation to nerve excitation (Rushton), 382.
Excitation in living material, discussion on transmission of (Pantin), 397.

Fishes, mechanism of the lateral sense organs of (Sand), 472.
Foster (J. S.) and Horton (C. A.) Quantitative spectrographic analysis of biological material II, 422.

Gamma-rays, action of on growing and on non-proliferating bacteria (Lea, Haines and Coulson), 1.
Gastric secretion and blood flow in hypophysectomized animals (Cutting, Dodds, Noble and Williams), 49.
Gastric secretion, effect of alterations in blood flow on (Cutting, Dodds, Noble and Williams), 39.

Haemoglobin, action of nitrite on in the absence of oxygen (Brooks), 368.
Haines (R. B.) *See* Lea, Haines and Coulson.
Haldane (J. B. S.) *See* Bell and Haldane.
Hallpike (C. S.) and Hartridge (H.) On the response of the human ear to audio-frequency electrical stimulation, 177.
Hartridge (H.) *See* Hallpike and Hartridge.
Haslewood (G. A. D.) *See* Bachmann and others.
Heterosis and the inheritance of quantitative characters (Ashby), 431.
Hewett (C. L.) *See* Bachmann and others.
Horton (C. A.) *See* Foster and Horton.

Lea (D. E.), Haines (R. B.) and Coulson (C. A.) The action of radiations on bacteria. III—γ-rays on growing and on non-proliferating bacteria, 1.
Leaves, nitrogenous constituents in starving (Yemm), 243.
Light of various wave-lengths, penetration into sea of (Poole and Atkins), 151.

Mayneord (W. V.) *See* Burrows, Mayneord and Roberts.
Medulla oblongata of Cyprinodont fishes, study of (Bhimacher), 59.
Menstrual cycle of the primates, X, XI (Zuckermann), 441, 457.
Metabolism, instantaneous measurement of muscle (Millikan), 218.
Millikan (G. A.) Experiments on muscle haemoglobin *in vivo*; the instantaneous measurement of muscle metabolism, 218.
Monochromatic rays entering the eye pupil at different points, luminous efficiency of (Stiles), 90.
Muscle haemoglobin *in vivo*, experiments on (Millikan), 218.

Neoplasia following the application of X-rays to inflammatory lesions (Burrows, Mayneord and Roberts), 213.
Nerve axoplasm, protein constituents of (Bear, Schmitt and Young), 520.
Nerve axoplasm, ultrastructure of (Bear, Schmitt and Young), 505.
Noble (R. L.) *See* Dodds, Noble, Scarff and Williams; and Cutting, Dodds, Noble and Williams.

Oestrogenic hormone, part played by in menstrual cycle (Zuckermann), 457.
Oestrone-threshold of the uterus of the Rhesus monkey (Zuckermann), 441.

Pantin (C. F. A.) Discussion on the transmission of excitation in living material, 397.
Pirie (N. W.) *See* Bawden and Pirie.
Pituitary control of alimentary blood flow and secretion (Dodds, Noble, Scarff and Williams; and Cutting, Dodds, Noble and Williams), 22, 27, 39, 49.
Polypores, wood-rotting, enzymes of (Bose and Sarkar), 193.
Poole (H. H.) and Atkins (W. R. G.) The penetration into the sea of light of various wave-lengths as measured by emission or rectifier photo-electric cells, 151.
Protein catabolism in starving leaves, (Yemm), 243.
Protein constituents of nerve axoplasm (Bear, Schmitt and Young), 520.

Respiration of barley plants, III (Yemm), 243.
Respiration, plant, VII, Analytic studies (Barnell), 321.
Roberts (J. E.) *See* Burrows, Mayneord and Roberts.
Robinson (Mrs A. M.) *See* Bachmann and others.
Rushton (W. A. H.) A graphical solution of a differential equation with application to Hill's treatment of nerve excitation, 382.

Sand (A.) The mechanism of the lateral sense organs of fishes, 472.
Sarkar (S. N.) *See* Bose and Sarkar.
Scarff (R. W.) *See* Dodds, Noble, Scarff and Williams.
Schmitt (F. O.) *See* Bear, Schmitt and Young.
Solanaceous plants infected with tobacco mosaic virus, liquid crystalline substances from (Bawden and Pirie), 274.
Squid, sheath components of the giant nerve fibres of (Bear, Schmitt and Young), 496.
Stiles (W. S.) The luminous efficiency of monochromatic rays entering the eye pupil at different points and a new colour effect, 90.
Stomach, changes in, produced by the administration of posterior pituitary extract (Dodds, Noble, Scarff and Williams), 22.

Thalamus, connexions of the arcuate nucleus of (Clark), 166.

Virus, tobacco mosaic (Bawden and Pirie), 27.
Visual sensitivity, change of with time (Crawford), 69.

Williams (P. C.) *See* Dodds, Noble, Scarff and Williams; and Cutting, Dodds, Noble and Williams.

Yemm (E. W.) The respiration of barley plants: III—Protein catabolism in starving leaves, 243.
Young (J. Z.) *See* Bear, Schmitt and Young.

Zuckermann (S.) The menstrual cycle of the primates: X—The oestrone-threshold of the uterus of the Rhesus monkey. XI—The part played by oestrogenic hormone in the menstrual cycle, 441, 457.

END OF THE ONE HUNDRED AND TWENTY-THIRD VOLUME (SERIES B)

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